ROLES OF FC RECEPTORS IN DISEASE AND THERAPY

EDITED BY: Latha P. Ganesan, Mark S. Cragg and Gestur Vidarsson
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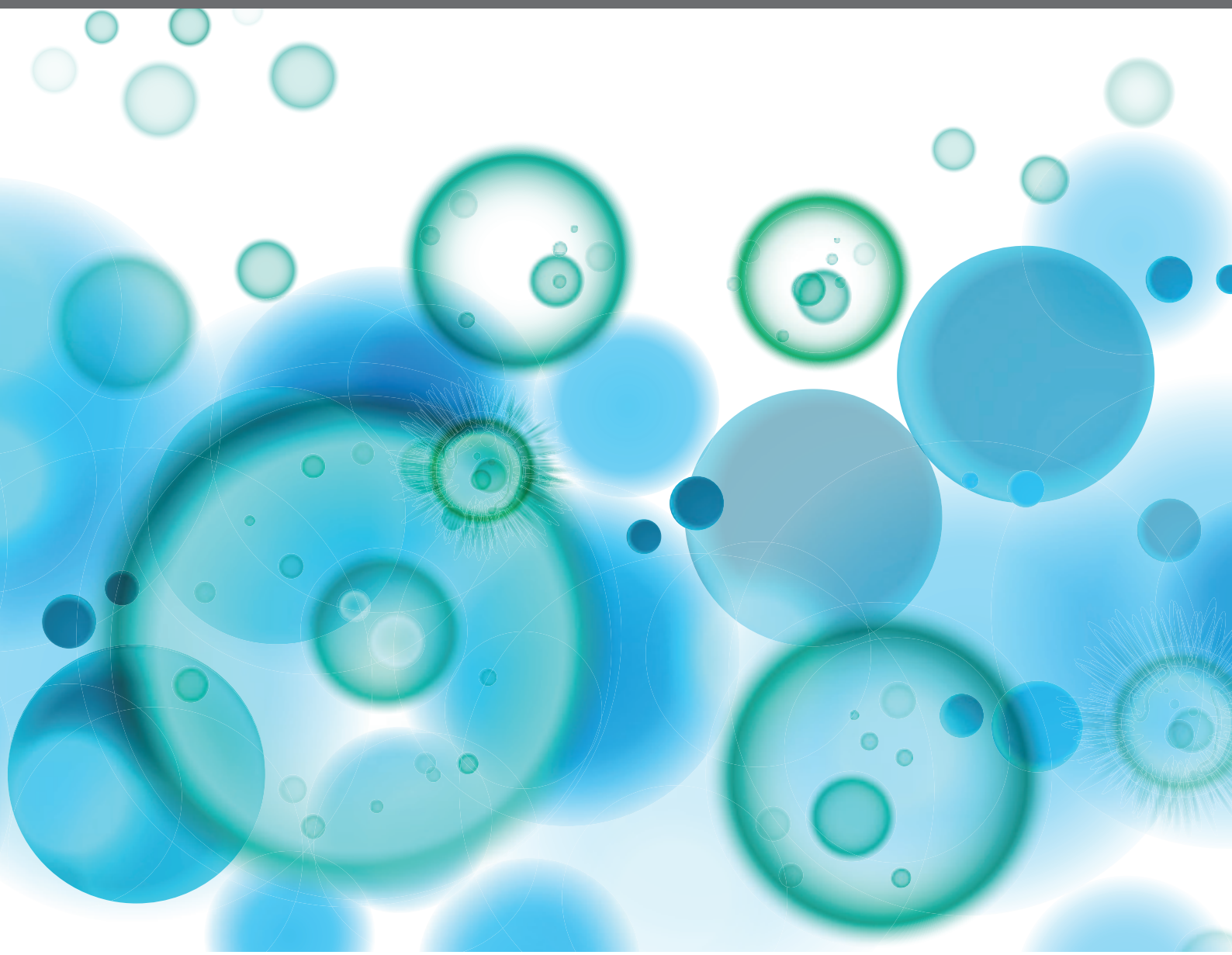

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ROLES OF FC RECEPTORS IN DISEASE AND THERAPY

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Table of Contents

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EDITORIAL
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Editorial: Roles of Fc Receptors in Disease and Therapy

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Keywords: antibody, Fc gamma receptor (FcγR), immune response, disease, therapy

Editorial on the Research Topic

Roles of Fc Receptors in Disease and Therapy

The humoral immune response is one of the central tenets of mammalian immunity. Delivered through the production of antibodies of multiple classes (IgG, IgM, IgA, IgD, and IgE) and sub-classes (e.g., IgG1, IgG2, IgG3, and IgG4 in humans) their activities are achieved through their inherent ability to bind with exquisite specificity to a given target antigen and then engage various immune effector functions to elicit the appropriate response. Chief amongst these are cellular immune effectors such as macrophages, NK cells, and neutrophils which are engaged through their expression of Fc receptors (FcR), binding the Fc portion of the immunoglobulins. Accordingly, different classes and isotypes of antibody engage a selection of different FcR. For example in the murine system there are receptors that are specific for IgG, IgM, IgE as well as receptors that are dually-specific for IgM and IgA with paralogues in human cells. A bewildering array of immune and non-immune cells express these various receptors in different combinations, leading to a highly complex system for regulating and evoking antibody responses. Various FcR evoke cellular activation (FcγRIIa and FcγRIIa), whereas others are inhibitory (FcγRIIb), with still others being capable of evoking intracellular transport and recycling of IgG (FcRn) to establish long serum half-lives. Clearly, careful regulation of expression, signaling and modulation is required for a healthy, well-functioning and balanced immune system. In this Research Topic, a series of articles are provided to reveal comprehensive insights on the role of these various FcR in health and disease, taking into account the wide spectrum of receptors and cells expressing them. Most importantly the insights presented in these articles pave the way for powerful immunotherapies and emerging principles about how FcR can be exploited for therapeutic purposes for various diseases, including infectious diseases, autoimmune diseases, and cancer.

In total, 6 original research articles were contributed on the various topics, spanning the genetics and function of the disparate FcγR. While Kerntke et al. revisited the question of the number and expression pattern of FcγR on myeloid cells, Nagelkerke et al. dissected the genetic variation within the family, including duplications and deletions within the low affinity FcγR-locus. How the GPI-linked FcγRIIIb affects tumor cell killing by PMN through therapeutic monoclonal antibodies is furthermore tackled by Treffers et al. while Kang et al. describes a new re-engineered IgG molecule that selectively engages FcγRIIIa-V158 for enhanced therapeutic benefit through a single FcγR. Brandsma et al. also investigated the differential capacity of tumor killing through FcR that engage different antibody isotypes, specifically addressing the role of FcαR vs. FcγR. Parameters affecting the function of FcRn were also tackled. Finally, Kendrick et al. mathematically modeled FcRn kinetics and suggest a novel reduced-order model based on a new expression for the fractional catabolic rate that can be used to predict plasma IgG responses.

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This Research Topic also features 18 Review Articles spanning these disparate areas. FcRn is tackled by Pyzik et al. and Nagelkerke et al. also contributes a comprehensive review of FcγRII-FcγRIII genetics. Anania et al. systematically discuss the structure-function relationship of FcγRII receptors, while the contribution of FcγRIIb in the development of autoimmune diseases in mouse models gets a comprehensive assessment by Verbeek et al.. Breedveld and van Egmond review pathologies and new opportunities resulting from targeting FcαR. In addition, Foss et al. extend the scope of this topic to the cytosolic FcR, TRIM21, while Liu et al. and Kubagawa et al. discuss the role of the IgM binding, FcμR in immunity. The role of FcR in infectious diseases and vaccine development is covered by Boudreau and Alter, discussing FcR and their role in the protection against influenza infection and future prospects to leverage FcR immune activity for the development of vaccines with Jenks et al. focusing on the subversion of immune responses by FcR encoded by Herpes simplex virus. The involvement of FcR in various inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, and immune thrombocytopenia with a focus on antibody-mediated autoimmunity is covered by Mkaddem et al.. This includes the mechanism of FcR-receptor-mediated inflammation and how to potentially exploit this knowledge therapeutically. Katsinelos et al. focuses on the role of antibodies and receptors involved in neurodegeneration during Alzheimer's and Parkinson's disease, while Castro-Dopico and Clatworthy discuss the role of FcR in inflammatory diseases of the gut, namely inflammatory bowel diseases. Patel et al. discusses the multiple variables that are at play in the interface between target and effector cells through IgG-FcγR engagement, with a focus on the largely undescribed role for FcγR-glycosylation in mediating the underlying recognition events. FcR signaling is also specifically covered by Gomez et al. for FcεRI in Allergic disease, including seasonal rhinitis, atopic dermatitis, urticaria, anaphylaxis, and asthma, while Koenderman et al. reviews how the activation status of FcR can be affected by inside-out signaling. Finally, the importance of FcR in cancer and cancer therapies, in particular, the role of checkpoint inhibitors therein, is given comprehensive review by Chen et al. and special focus on FcγRIIb mediated antitumor immunity by Teige et al.

Overall, it is clear that the knowledge acquired from the articles contained within this special issue highlights the complexity of the FcR family and their importance in multiple aspects of health and disease. However, equally clear is the fact that this family of receptors, despite being investigated for over 4 decades, still harbors many secrets, reinforcing that we still lack a complete understanding of their complex regulation, interaction and impacts. As central to humoral immunity, modulating disease pathogenesis and acting as a key determinant of antibody therapeutics, it is also similarly evident that further research in this area is still warranted. We look forward to seeing what the intensive study of these receptors shows in the coming decade.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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FcγRIIIb Restricts Antibody-Dependent Destruction of Cancer Cells by Human Neutrophils

Louise W. Treffers[1], Michel van Houdt[1], Christine W. Bruggeman[1], Marieke H. Heineke[2], Xi Wen Zhao[1], Joris van der Heijden[1], Sietse Q. Nagelkerke[1,3], Paul J. J. H. Verkuijlen[1], Judy Geissler[1], Suzanne Lissenberg-Thunnissen[1], Thomas Valerius[4], Matthias Peipp[4], Katka Franke[1], Robin van Bruggen[1], Taco W. Kuijpers[1,3], Marjolein van Egmond[2], Gestur Vidarsson[1], Hanke L. Matlung[1†] and Timo K. van den Berg[1,2†]*

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The function of the low-affinity IgG-receptor FcγRIIIb (CD16b), which is uniquely and abundantly expressed on human granulocytes, is not clear. Unlike the other Fcγ receptors (FcγR), it is a glycophosphatidyl inositol (GPI) -anchored molecule and does not have intracellular signaling motifs. Nevertheless, FcγRIIIb can cooperate with other FcγR to promote phagocytosis of antibody-opsonized microbes by human neutrophils. Here we have investigated the role of FcγRIIIb during antibody-dependent cellular cytotoxicity (ADCC) by neutrophils toward solid cancer cells coated with either trastuzumab (anti-HER2) or cetuximab (anti-EGFR). Inhibiting FcγRIIIb using CD16-F(ab')$_2$ blocking antibodies resulted in substantially enhanced ADCC. ADCC was completely dependent on FcγRIIa (CD32a) and the enhanced ADCC seen after FcγRIIIb blockade therefore suggested that FcγRIIIb was competing with FcγRIIa for IgG on the opsonized target cells. Interestingly, the function of neutrophil FcγRIIIb as a decoy receptor was further supported by using neutrophils from individuals with different gene copy numbers of *FCGR3B* causing different levels of surface FcγRIIIb expression. Individuals with one copy of *FCGR3B* showed higher levels of ADCC compared to those with two or more copies. Finally, we show that therapeutic antibodies intended to improve FcγRIIIa (CD16a)-dependent natural killer (NK) cell ADCC due to the lack of fucosylation on the N-linked glycan at position N297 of the IgG$_1$ heavy chain Fc-region, show decreased ADCC as compared to regularly fucosylated antibodies. Together, these data confirm FcγRIIIb as a negative regulator of neutrophil ADCC toward tumor cells and a potential target for enhancing tumor cell destruction by neutrophils.

Keywords: FcγRIIIb, neutrophil, ADCC, cancer, granulocyte, Fc-receptor, CNV, glycoengineering

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INTRODUCTION

Fc-receptors play a vital role in cancer immunotherapy by inducing ADCC and antibody dependent cellular phagocytosis (ADCP). Most cancer targeting therapeutic antibodies currently on the market are of the IgG class, and thus human FcγRs constitute the key receptors for ADCC during cancer immunotherapy (1). The principal FcγR receptor on neutrophils required for mediating ADCC of solid cancer cells appears to be FcγRIIa (2, 3), with ∼30–60-thousand copies expressed per cell (4), sometimes in combination with the activating receptor FcγRIIc, present on a minority of about 15–20% of Caucasian individuals (5). The high affinity receptor FcγRI (CD64) is only present on activated neutrophils, but does generally not contribute to ADCC of solid cancer cells even when expressed (3). Both FcγRI and FcγRIIa signal via immunoreceptor tyrosine-based activation motifs (ITAM), encoded in the cytoplasmic tail of the receptors (FcγRIIa) or in the associated γ-chain (FcγRI). Lastly, neutrophils express the highly abundant, 100–200-thousand copies per cell, low affinity receptor FcγRIIIb, which is a GPI-linked Fc-receptor that lacks intrinsic intracellular signaling capacity (4). This receptor is selectively present on neutrophils and on a subset of basophils (6). In spite of the lack for direct signaling through FcγRIIIb evidence from a number of studies show that FcγRIIIb cooperates together with other FcγR in the context of the phagocytosis of opsonized microbes (7). This suggests that the abundantly expressed FcγRIIIb primarily acts to facilitate enhanced recognition and that ITAM signaling via the other FcγR, in particular FcγRIIa, is sufficient, or at least instrumental, to trigger the phagocytic process. The FcγRIIIb-encoding gene, *FCGR3B,* which only occurs in humans and certain primates (8), is located within the *FCGR2/3* locus on human chromosome 1, where it is prone to gene copy number variation (CNV) (9). The CNV of *FCGR3B* ranges from very rare individuals with no *FCGR3B,* to individuals with five copies of this gene (10). *FCGR3B* CNV has been shown to affect various diseases, i.e., a low CNV of *FCGR3B* was shown to result in an increased susceptibility to autoimmune diseases like systemic lupus erythematosus (SLE) (11, 12), primary Sjogren's syndrome (pSS) (12), Wegener's granulomatosis (WG) (12) and rheumatoid arthritis (RA) (13). A high CNV of *FCGR3B* has been associated with psoriasis vulgaris in Han Chinese (14). Nevertheless, no enhanced susceptibility to bacterial or fungal infection was observed in very rare individuals lacking FcγRIIIb expression (15), also showing that their neutrophils were able to function normally in regards to phagocytosis and superoxide generation (16). In addition, several polymorphic variants of the *FCGR3B* gene, known as the NA1, NA2, and SH haplotypes exist (17, 18), which do not result in marked differences in IgG-affinity. On the level of neutrophil-mediated ADCC of cancer cells all polymorphic variants appear similarly effective (3), but neutrophils from NA1NA1 individuals have been reported to bind and phagocytose IgG-opsonized bacteria and red cells somewhat more effectively than their heterozygous NA1NA2 and homozygous NA2NA2 counterparts (19, 20).

Neutrophils constitute a major first line of host immune defense against fungal and bacterial infection (21). After extravasation from blood circulation they can enter a variety of tissues, including solid tumors (22–25). And even though the role of neutrophils in cancer is complex, with evidence for both positive or negative effects on tumor development (26), it is clear that neutrophils can contribute to the destruction of cancer cells particularly upon treatment with cancer therapeutic antibodies, as demonstrated now in a variety of animal models (27–30). Recently, we have found that neutrophils destroy antibody-opsonized cancer cells by a unique cytotoxic mechanism, termed *trogoptosis*, where neutrophils take up small pieces of cancer cell membrane, which leads to mechanical injury of the plasma membrane of cancer cells causing necrotic cell death (31). This neutrophil-mediated cytotoxic process can further be enhanced by inhibiting the interaction between the innate inhibitory immunoreceptor signal regulatory protein α (SIRPα) and CD47 (31–33). SIRPα is specifically expressed on myeloid cells and interacts with its ligand CD47, which is expressed ubiquitously, and is often overexpressed on cancer cells, acting as a "don't eat me" signal to prevent phagocytosis by macrophages (33–35). Interference with CD47-SIRPα interactions has also been shown to increase ADCC by monocytes and neutrophils, making this interaction an innate immune checkpoint and an attractive target for enhancing antibody therapy in cancer (32, 33, 36). Obviously, it is of interest to identify other pathways that negatively impact neutrophil ADCC.

Even though FcγRIIIb is a very abundant protein on neutrophils (37), its actual function has remained uncertain. Available evidence in the context of phagocytosis of antibody-opsonized bacteria by human neutrophils suggests that FcγRIIIb cooperates with activating FcγR, like FcγRIIa/c, to promote phagocytosis (7, 38–40), and we have confirmed this in the current study. However, here we show that with respect to neutrophil mediated ADCC, FcγRIIIb rather acts as a decoy receptor for IgG, likewise competing with FcγRIIa for the binding of therapeutic antibodies, thereby resulting in decreased ADCC. Thus, in the context of cancer FcγRIIIb on neutrophils uniquely functions as a limiting factor, thereby identifying it a as potential target for enhancing the therapeutic efficacy of cancer therapeutic antibodies.

MATERIALS AND METHODS

Cells and Culture

The HER2/Neu-positive human breast cancer carcinoma cell line SKBR3 (ATCC) was cultured in IMDM medium (Gibco) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO_2. SKBR3-CD47KD cells were generated by lentiviral transduction of pLKO.1-puro—CD47KD (5′ ccgggcacaattacttgga ctagttctcgagaactagtccaagtaattgtgcttttt 3′), resulting in a CD47 expression of 10–15% of the parental cell line according to

Abbreviations: FcγR, Fcγ receptor; ADCC, antibody dependent cellular cytotoxicity; NK cell, natural killer cell; ADCP, antibody dependent cellular phagocytosis; ITAM, immunoreceptor tyrosine-based activation motif; CNV, copy number variation; G-CSF, granulocyte-colony stimulating factor; IFNγ, interferon-γ.

instructions provided by the manufacturer (Sigma), as show previously (32). Transduced cells were selected with 1 μg/mL of puromycin. As control cell line, empty vector shRNA were used (SKBR3-SCR). The CD47 knockdown cell line was routinely verified by flow cytometry.

The EGFR-positive human epidermoid carcinoma cell line A431 (ATCC) was cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO_2. A431-CD47KO cell lines were generated by lentiviral transduction of pLentiCrispR-v2—CD47KO [pLentiCrispR-v2 was a gift from Feng Zhang (Addgene plasmid #52961)], using 5′ cagcaacagcgccgctacca 3′ as the CD47 CrispR target sequence. Transduced cells were selected with 1 μg/mL of puromycin, followed by limiting dilution. A clone lacking CD47 expression was selected by flow cytometry. An A431-SCR cell line was used as control for the CD47KO generated using CRISPR-Cas9 technology using a scrambled vector.

Neutrophil Isolation

Neutrophils from healthy donors were isolated as previously described (41). In short, granulocytes were isolated from blood by density gradient centrifugation (2,000 rpm, 20 min, 20°C) with isotonic Percoll (1.069 g/mL) and erythrocyte lysis. The pellet fraction was lysed with ice-cold NH4Cl (155 mmol/LNH,CI, 10 mmol/L KHCO, 0.1 mmol/L EDTA, pH 7.4) solution for 5–10 min to destroy erythrocytes. Cells were centrifuged at 4°C (1,500 rpm, 5 min), and residual erythrocytes were lysed for another 5 min. After this, granulocytes were washed twice in cold phosphate buffered saline (PBS) containing HSA (0.5% wt/vol).Isolated neutrophils were used at a concentration of 5×10^6 cells/mL. Cells were cultured in $HEPES^+$ medium (containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.2 mM K_2HPO_4, 20 mM Hepes, 5.5 mM glucose, and 0.5% HSA), in the presence of 10 ng/ml clinical grade G-CSF (Neupogen; Amgen, Breda, The Netherlands) and 50 ng/mL recombinant human interferon-γ (Pepro Tech Inc, USA) at a concentration of 5×10^6 cells/mL for 4 or 16 h. After 16 h, cell viability was determined by the percentage of FITC-Annexin V (BD Pharmingen, San Diego, CA) positive cells on FACS, after which the cell concentration was corrected to 5×10^6 viable cells/mL. Cells were consequently washed and prepared for analysis by ADCC assay. All blood was obtained after informed consent and according to the Declaration of Helsinki principles (version Seoul 2008).

Antibodies and Reagents

FcγR expression was determined on FACS and depicted as MFI (median fluorescent intensity) using the following antibodies: anti-human FcγRI (Clone 10.1, mouse IgG1, BD Pharmingen, San Diego, CA), anti-human FcγRIIa (Clone AT10, mouse IgG1, AbD Serotec, Oxford, U.K.), anti-human FcγRIIIb (Clone 3G8, mouse IgG1, BD Pharmingen, San Diego, CA), all FITC labeled. FcγRs antagonistic antibodies were used in ADCC and trogocytosis assays at a final concentration of 5 μg/mL: monovalent human Fc fragments (Bethyl, USA) for blocking FcγRI as used previously (3), anti-human CD32 $F(ab')_2$ (Clone 7.3, Ancell) to block FcγRIIa/b/c, anti-human CD16 $F(ab')_2$ (Clone 3G8, Ancell) to block FcγRIIIa/b at a concentration of 10 μg/mL. CD11b expression was determined with the FITC labeled anti-CD11b antibody (Lot 8000236273, Pelicluster), and SIRPα with the FITC labeled mouse IgG_1 antibody 12C4, previously described in (32). FITC-labeled mouse IgG1 was used as isotype control (Pelicluster). Afucosylated trastuzumab was generated in our laboratory as described before for afucosylated rituximab (42). Briefly, CHO-KI or Lec13 cells were transfected with antibody LC and HC expression constructs using transfection kit V from the Amaxa Nucleofectior System (Lonza, Cologne, Germany). The medium was exchanged by culture medium after 48 h, which contained 500 μg/mL hygromycin B. Single-cell subclones were created by limiting dilution. The produced antibodies were purified from the cell culture supernatant using CaptureSelect^{TM} IgG-CH1 Affinity Matrix (Thermo Fisher Scientific). IgA2-HER2 was generated by synthesizing (IDT, Leuven, Belgium) the variable heavy and light chain V gene encoding for trastuzumab (sequence as obtained from https://www.drugbank.ca/) and cloning into pcDNA3.1 expression vectors encoding for the constant regions for IgA2 and kappa, respectively, as described previously (43). The resulting expression vectors were then used to produce the antibodies in HEK Freestyle cells as we described previously (44, 45). Briefly, after transfection, cell supernatant was harvested after 5 days, after which cells were centrifuged (≥4000 g) and the supernatant was filtered using a 0.45 nm puradisc syringe filter (Whatmann, GE Healthcare, 10462100). Antibody concentration was determined via enzyme-linked immunosorbert assay (ELISA), as described previously (46). To create afucosylated antibodies, the decoy substrates for fucosylation, 2-deoxy-2-fluoro-l-fucose (2FF) (Carbosynth, MD06089) were added 4 h post transfection. Similarly, human anti-pneumococcal serotype 6B Gdob1 antibodies (IgG_1) (47, 48), regular and afucosylated were produced in the same system (44, 45). They were used at a concentration of 10 μg/mL throughout the experiment to opsonize *S. pneumoniae*. Polyclonal human IgG (IVIG, nanogam, Sanquin) was used to opsonize *S. aureus* at a concentration of 1 mg/mL) for 10 min at 37°C.

ADCC

Cancer cell lines were labeled with 100 μCi ^{51}Cr (Perkin-Elmer) for 90 min at 37°C. After 3 washes with PBS, 5×10^3 cells were incubated in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin for 4 h at 37°C and 5% CO_2 in a 96-wells U-bottom plate together with neutrophils in a E:T ratio of 50:1 in the presence of 5 μg/mL therapeutic antibody. After the incubation supernatant was harvested and analyzed for radioactivity using a gamma counter (Wallac). The percentage of cytotoxicity was calculated as [(experimental cpm- spontaneous cpm)/ (total cpm–spontaneous cpm)] × 100%. All conditions were measured in triplicate.

Trogocytosis Assay

To determine the amount of tumor membrane taken up by neutrophils a FACS based assay was used. Cancer cells were labeled with a lipophilic membrane dye (DiO, 5 μM, Invitrogen)

for 30 min at 37°C. After washing the target cells with PBS they were incubated with neutrophils in a U-bottom 96-wells plate at a E:T ratio of 5:1 in the absence or presence of 0.5 μg/mL therapeutic antibody. Samples were fixed with stopbuffer containing 0.5% PFA, 1% BSA and 20 mM NaF and measured by flow cytometry. After gating for neutrophil population, the mean fluorescent intensity (MFI) and the percentage of cells positive for DiO were determined.

Bacterial Phagogytosis

Uptake of FITC labeled *S. aureus* was performed in a 96 wells plate for 15 min at 37°C shaking, with 0.5×10^6 neutrophils and 25×10^6 bacteria in a final volume of 250 μL in $HEPES^+$ medium. Bacteria were opsonized with polyclonal IgG (IVIG) (1 mg/mL) for 10 min at 37°C. Cells were fixed with stopbuffer (0.5% PFA, 1% BSA, 20 mM NaF) for 30 min at 4°C and measured by flow cytometry (BD FACSCanto II). Uptake of Dy488 labeled heat killed *Streptococcus Pneumoniae* was performed in a 96 wells plate for 30 min at 37°C while shaking, with 1.5×10^4 neutrophils and 5×10^6 bacteria in a final volume of 225 μL in $HEPES^+$ medium. When applicable, neutrophils were incubated with FcγR blockers for 15 min at RT. Bacteria were opsonized with GDob1 antibody at a concentration of 10 μg/mL throughout the experiment. Cells were fixed with stopbuffer (0.5% PFA, 1% BSA, 20 mM NaF) for 30 min at 4°C and measured by flow cytometry (BD FACSCanto II).

MLPA

Genotyping of individuals for *FCGR3B* CNV was performed using the *FCGR*-specific Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRC Holland), using genomic DNA isolated from whole blood with the QIAamp® kit (Qiagen, Hilden, Germany). The MLPA assay was performed as described previously (49). In brief, 5 μL of DNA (20 ng/μL) was denatured at 98°C for 5 min and subsequently cooled to 25°C in a thermal cycler with heated lid; To each sample 1.5 μL buffer and 1.5 μL buffer probe mix were added and incubated for 1 min at 95°C, followed by 16 h at 60°C. After this, 32 μL of ligase-65 mix was added to each sample at 54°C, followed by an incubation of 15 min at 54°C and 5 min at 98°C, followed by a 4 times dilution of the ligation mixture. This was followed by addition of 10 μL of polymerase mix, which contained one single primer pair, after which the polymerase chain reaction (PCR) was started immediately. PCR conditions were 36 cycles of 30 s at 95°C, 30 s at 60°C, and 60 s at 72°C, followed by 20 min at 72°C. After the PCR reaction, 1 μL of the PCR reaction was mixed with 0.5 μL CXR 60–400 (Promega, Madison, WI) internal size standards and 8.5 μL deionized formamide, and the mixture was incubated for 10 min at 90°C. The products were then separated by electrophoresis on an ABI-3130XL (Applied Biosystems, Foster City, CA).Data were analyzed using GeneMarker v1.6 sofware.

IL-8 ELISA

IL-8 production was measured using the Human IL-8 ELISA Ready-SET-Go! (2nd Generation) kit (eBioscience, Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Wavelengths were measured with an iMark microplate absorbance reader (Bio-rad Laboratories, Hercules, CA).

Study Approval

The study was performed according to national regulations with respect to the use of human materials from healthy, anonymized volunteers with written informed consent, and the experiments were approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam according to the Declaration of Helsinki principles (version Seoul 2008).

Data Analysis and Statistics

Statistical differences were determined by either paired or ordinary one way ANOVA, with Sidak or Dunnett's *post-test*, or by paired student's *t*-test, as indicated in the figure legend.

RESULTS

We studied the role of FcγRIIIb during neutrophil ADCC toward solid cancer cells. Although FcγRIIIb is apparently unable to signal by itself, it definitely has the capacity to bind IgG and as such could potentially influence responses via other activating Fcγ-receptors on neutrophils, in either a positive or negative fashion. Such activating FcγRs present on neutrophils include FcγRI, only present after neutrophil activation, and FcγRIIa, which appears to be the main receptor required for ADCC against cancer cells expressing the tumor antigens HER2/Neu or EGFR (**Supplementary Figure 1**) (2, 3). Neutrophil-mediated ADCC toward cancer cells can be enhanced after neutrophil-activation, e.g., by granulocyte-colony stimulating factor (G-CSF) and interferon-γ (IFNγ) (32). This stimulation causes a change in the expression levels of the FcγRs, resulting in expression of FcγRI, a small decrease in expression in FcγRIIa, and, of particular interest, a substantial decrease in FcγRIIIb (**Figure 1A**). The reduction in FcγRIIIb expression could well be due to cleavage of FcγRIIIb by protease release after neutrophil activation (50, 51). As mentioned before FcγRIIIb is subject to considerable gene CNV (9), and the expression levels of FcγRIIIb are directly linked to the number of copies present in the genome (**Figure 1B**). Upon stimulation FcγRIIIb levels on neutrophils are gradually reduced and the variation among individuals with different FcγRIIIb levels are essentially blunted (**Figure 1B**).

FcγRIIIb Functions as a Decoy Receptor During Neutrophil ADCC

We first determined the effect of blocking FcγRIIIb with $F(ab')_2$ fragments of specific anti-FcγRIIIb on both their ability to take up cancer cell fragments (trogocytosis) (**Figure 2A**) as well as their cytotoxic capacity, as measured by ^{51}Cr-release. The use of $F(ab')_2$-fragments is absolutely critical here as intact anti-FcγR antibodies may also exert non-specific blocking by the so called "Kurlander" phenomenon (52). Blocking FcγRIIIb resulted in a prominent increase in both trogocytosis and ADCC, clearly suggesting that FcγRIIIb plays a negative role in neutrophil-mediated antibody-dependent destruction of cancer cells (**Figures 2B–J**). Similar results were obtained

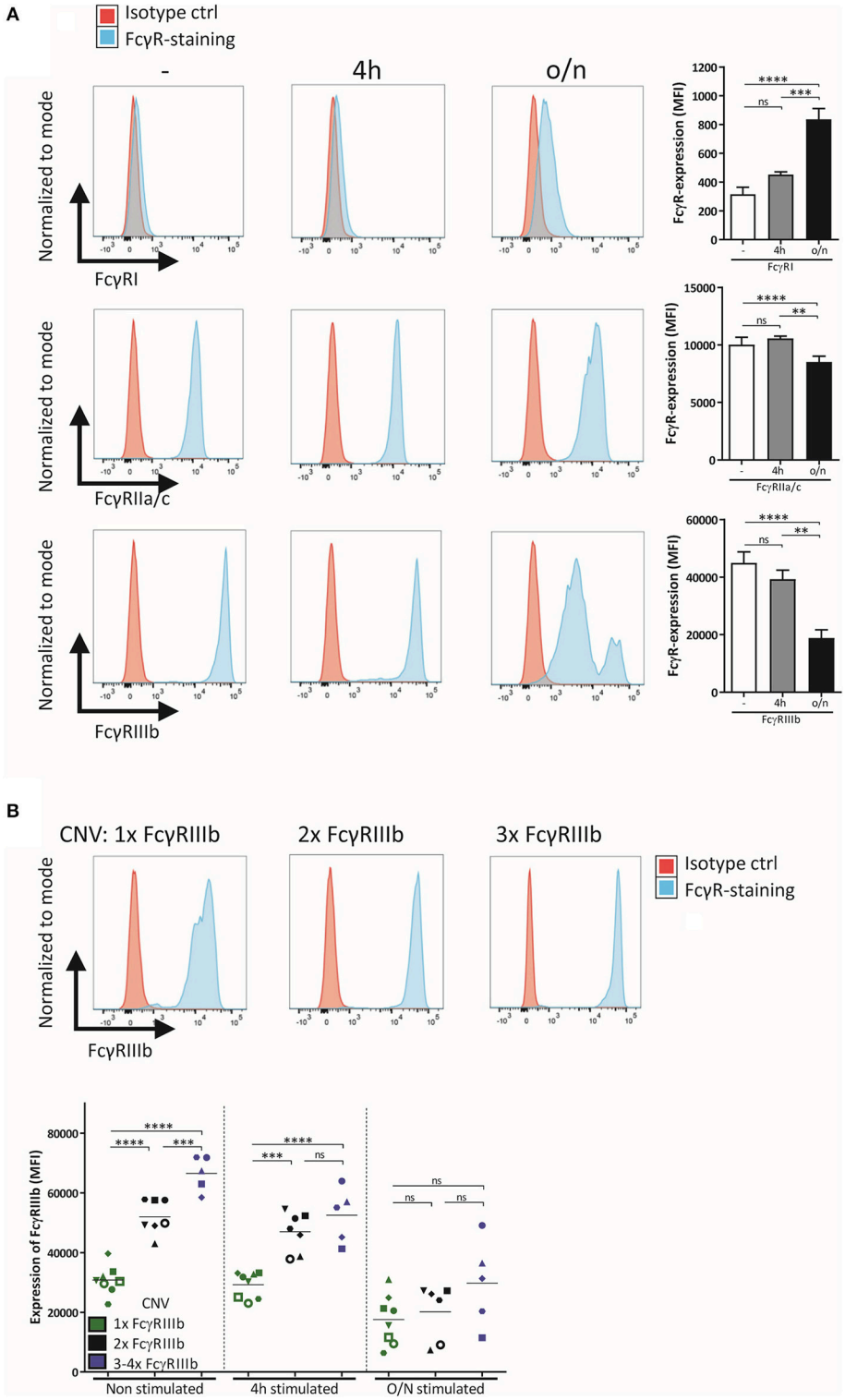

FIGURE 1 | Expression of FcγR on neutrophils depends on activation status and CNV. **(A)** FcγR expression, shown as representative histograms and bar graphs (MFI), was determined for freshly isolated neutrophils and neutrophils stimulated for 4 h or overnight with G-CSF and IFNγ. **(B)** Neutrophils were isolated from donors with different copy numbers of *FCGR3B,* and their FcγRIIIb expression was checked using flow cytometry, before stimulation, after 4 h stimulation and after overnight stimulation with G-CSF and IFNγ. Individuals with only one copy of *FCGR3B* are represented by green dots, donors with two copies by black dots, donors with three or more copies of *FCGR3B* by blue dots. Each symbol represents an individual donor per color. Data shown are mean + SEM **(A)** and mean **(B)** with $N = 20$ **(A)** and $N = 5$–8 **(B)**, statistical analysis was performed by one-way paired ANOVA with Tukey *post-test*. ns, non-significant; $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

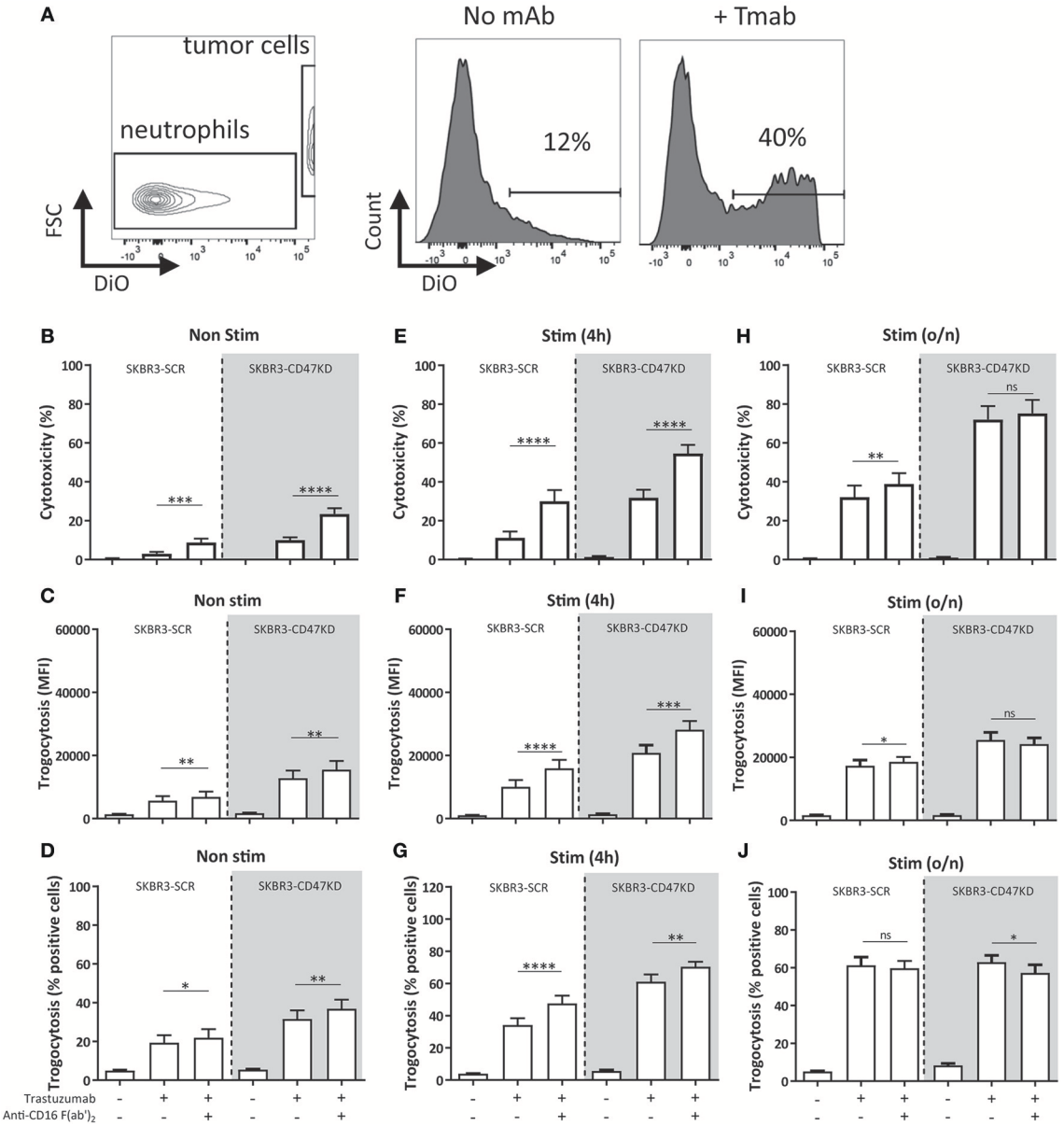

FIGURE 2 | Inhibition of FcγRIIIb results in increased ADCC and trogocytosis. **(A)** Gating strategy for FACS-based trogocytosis assay. Histograms show neutrophil population becoming positive for DiO after incubation with trastuzumab coated SKBR3-scrambled (SKBR3-SCR) cells. **(B–J)** Blocking FcγRIIIb increases ADCC and trogocytosis of trastuzumab coated SKBR3-SCR cells (white background) when using non-stimulated **(B–D)**, 4 h stimulated **(E–G)** and to a lesser extent overnight **(H–J)** stimulated neutrophils (with G-CSF and IFNγ). This effect is also present when inhibiting CD47-SIRPα interactions by CD47 knock-down (SKBR3-CD47KD, gray background). Data shown are means + SEM with **(B)** $N = 26$, **(C)** $N = 20$, **(D)** $N = 20$, **(E)** $N = 17$, **(F)** $N = 18$, **(G)** $N = 18$, **(H)** $N = 14$, **(I)** $N = 8$, **(J)** $N = 8$, statistical analysis was performed by paired *t*-test. ns, non-significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

when using other solid cancer cells, such as the EGFR-positive A431 cell line combined with the therapeutic antibody cetuximab (**Supplementary Figures 2A-C**). However, with both tumor targets, this negative role of FcγRIIIb was only visible when using freshly isolated neutrophils or neutrophils that had only been briefly stimulated (i.e., for 4 h; **Figures 2E–G**), when relatively large quantities of FcγRIIIb are still present on the neutrophil cell surface (see **Figure 1**). In contrary, when evaluated after overnight stimulation with G-CSF and IFNγ both neutrophil trogocytosis and ADCC were higher and the effect of FcγRIIIb blocking eventually disappeared (**Figures 2H–J**), which could be explained, at least in part, by the observed reduction in FcγRIIIb surface expression (**Figure 1**). Of interest, under these conditions the enhancing effect of CD47-SIRPα interference on cytotoxicity was still clearly visible (**Figure 2H**). Whether or not FcγRIIIb is highly

expressed on neutrophils, FcγRIIa remains the primary receptor responsible for triggering ADCC (**Supplementary Figure 1**). Thus, the principal FcγR mediating trogocytosis and subsequent ADCC of antibody-opsonized solid cancer cells by human neutrophils is FcγRIIa/c, and FcγRIIIb appears to function as a decoy receptor that apparently competes with FcγRIIa/c for binding to the Fc-portion of the opsonizing cancer therapeutic antibody.

To mimic checkpoint inhibitor blockade, we used SKBR3 cells with shRNA-knock-down for CD47 (reduction by ∼85–90%) (SKBR3-CD47KD) to inhibit the interactions between CD47 and SIRPα (32, 33, 36). The effect of inhibiting both CD47-SIRPα interactions and FcγRIIIb became even more apparent (**Figure 2**, gray background), also indicating that disruption of CD47-SIRPα and FcγRIIIb blockade were not part of the same inhibitory pathway and that such interferences could generate additive effects.

We hypothesized that blocking of FcγRIIIb could perhaps be resulting in increased production of IL-8 by neutrophils, which was previously described to occur when crosslinking FcαRI on neutrophils (53). The use of IgA therapeutic antibodies enhances neutrophil-mediated ADCC of cancer cells compared to using IgG antibodies (54, 55), which could be in part due to the production of cytokines, such as IL-8, by the neutrophils. We therefore determined the presence of IL-8 in the supernatant after neutrophil-mediated ADCC of SKBR3 cells in the presence or absence of FcγRIIIb blocking antibodies. The IL-8 levels that were produced using an anti-HER2 IgG antibody were significantly lower compared to IgA, as reported before (45),

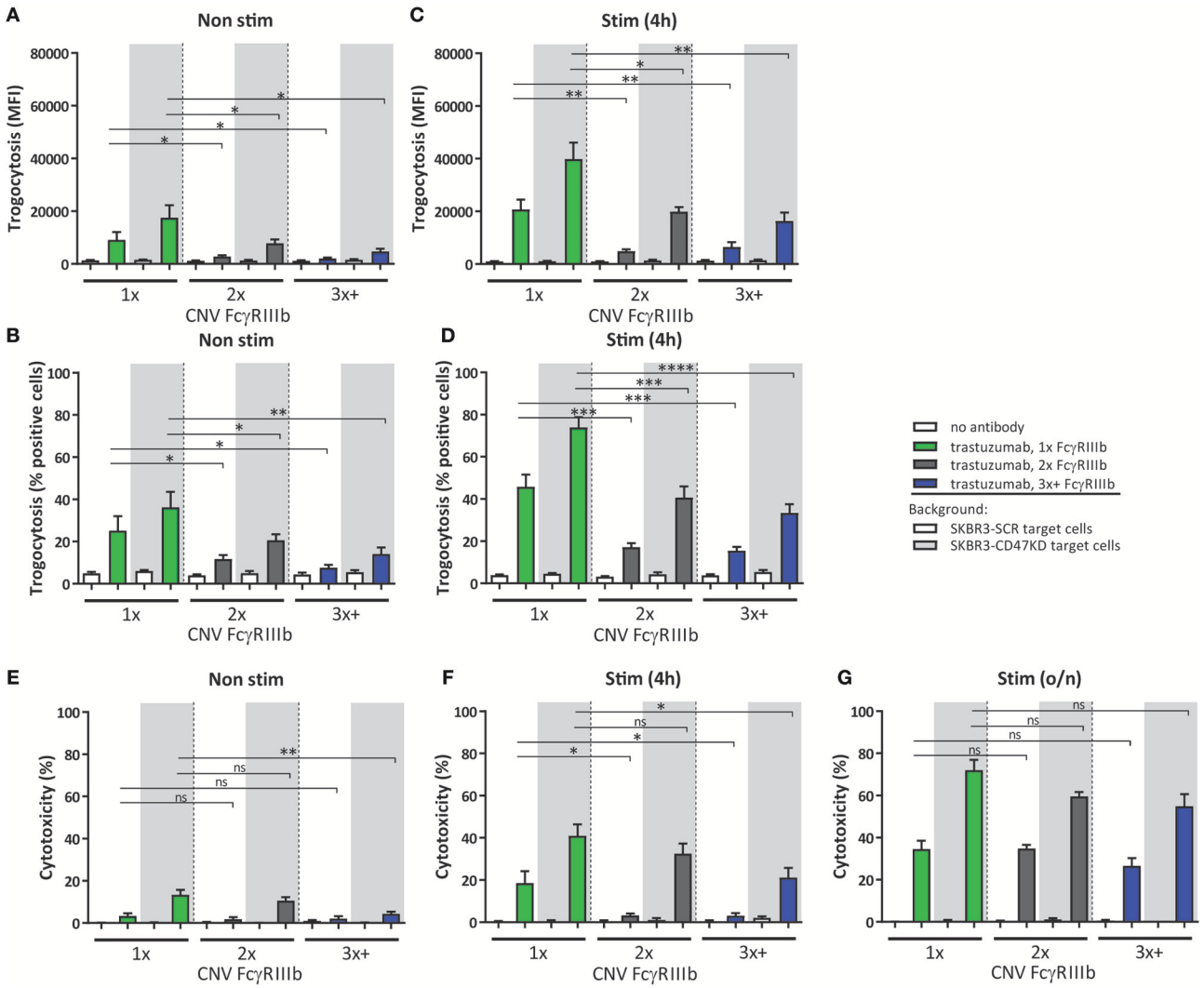

FIGURE 3 | CNV of *FCGR3B* affects ADCC of cancer cells by neutrophils. Trogocytosis **(A–D)** and ADCC **(E–G)** was determined for neutrophils from donors with various copies of *FCGR3B*. Freshly isolated **(A,B,E)**, 4 h stimulated **(C,D,F)** or overnight stimulated **(G)** neutrophils were combined with trastuzumab coated SKBR3-SCR (white background) or SKBR3-CD47KD cells (gray background). Shown are results from individuals with one copy (green), two copies (gray), or three or more copies (blue) of *FCGR3B*. Data shown are means + SEM with results from multiple experiments with donors ranging from $N = 7–10$. Statistical analysis was performed by one-way paired ANOVA with Sidak *post-test*. ns, non-significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

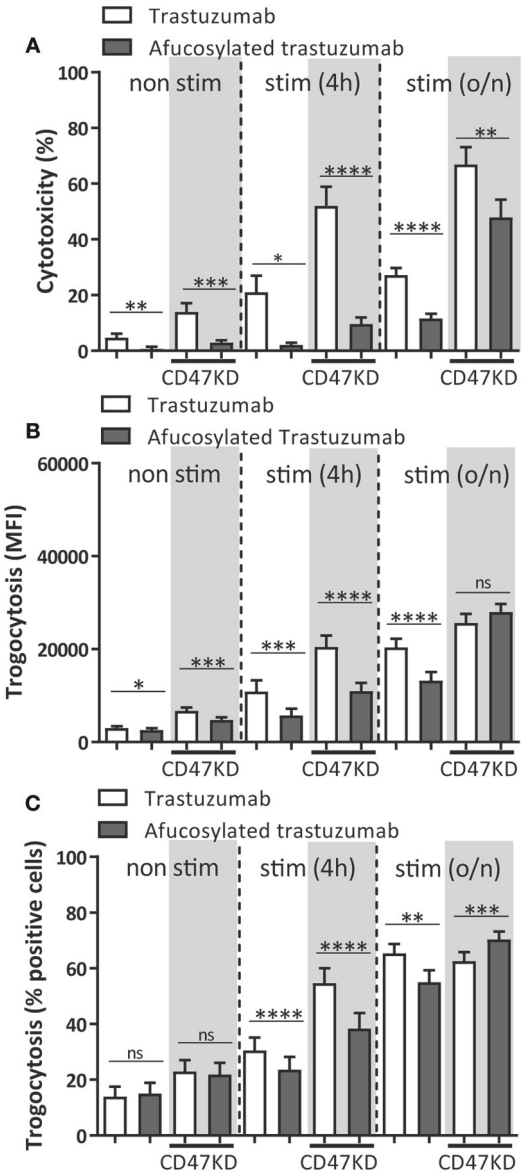

FIGURE 4 | Neutrophil ADCC and trogocytosis are reduced when afucosylated therapeutic antibodies are used. Afucosylated trastuzumab was compared to regularly fucosylated trastuzumab in both trogocytosis **(A,B)**, and ADCC **(C)**. Freshly isolated, 4 h and overnight stimulated (with G-CSF and IFNγ) neutrophils were combined with IgG opsonized SKBR3-SCR (white background) or SKBR3-CD47KD cells (gray background). Data shown are means + SEM with results from multiple experiments with donors ranging from $N = 9$–16. Statistical analysis was performed by paired *t*-test. ns, non-significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

and additional inhibition of FcγRIIIb showed no enhanced production of IL-8 (**Supplementary Figure 2D**).

FCGR3B CNV Determines Neutrophil ADCC

As indicated above FcγRIIIb surface expression on neutrophils is subject to considerable variation, and this is largely caused by gene copy number variation within the *FCGR2/3* locus (15). This enabled us to further study the observed negative contribution of FcγRIIIb to neutrophil ADCC. We therefore evaluated neutrophils from individuals with different copy numbers of *FCGR3B* determined by MPLA-based genotyping (49). Indeed, individuals with one copy of the gene have significantly increased ADCC and trogocytosis capacity compared to individuals with 2 or 3 or more copies, using neutrophils either freshly isolated or after 4 h stimulation with G-CSF and IFNγ (**Figure 3**). However, after overnight stimulation this difference essentially disappeared in all tested individuals and irrespective of *FCGR3* gene copy number (**Figure 1**). When comparing individuals with low (1x) and high FcγRIIIb (2–4x) expression blocking of FcγRIIIb could enhance ADCC to indistinguishable levels (**Supplementary Figure 3**), demonstrating that the difference in ADCC capacity between individuals with different *FCGR3B* CNV can indeed largely be attributed to the difference in FcγRIIIb expression on neutrophils. In these experiments the levels of other surface molecules relevant in the context of neutrophil ADCC (31, 32), including FcγRs, integrins or SIRPα were similar in all donors with different copy numbers of *FCGR3B* (**Supplementary Figure 4**).

When correlating the FcγRIIIb expression to either trogocytosis or ADCC capacity of neutrophils, irrespective of *FCGR3B* genetic status, we also noted a significant inverse correlation, but as expected this occurred only when using either freshly isolated neutrophils (**Supplementary Figures 5A-C**) or 4 h (**Supplementary Figures 5D-F**) stimulated neutrophils, but this correlation disappeared upon overnight neutrophil stimulation (**Supplementary Figures 5G-I**) consistent with the loss of surface FcγRIIIb. By comparison, we did not find any significant correlations when comparing FcγRIIa expression levels and killing (**Supplementary Figure 6**). These findings show that in non-stimulated neutrophils *FCGR3B* CNV is an important determinant of ADCC capacity, with higher levels of CNV and concurrent FcγRIIIb surface expression negatively affecting neutrophil ADCC, thereby providing genetic evidence for a role of FcγRIIIb as a decoy receptor.

Antibody Afucosylation Negatively Impacts Neutrophil ADCC

A number of mutations and posttranslational modifications of therapeutic antibodies have previously been explored for the purpose of improving their clinical potential. One of these alterations is antibody afucosylation, which changes the glycan linked to asparagine at position 297 (N297). Afucosylation of this glycan increases the binding affinity of the antibody to FcγRIIIa (44, 56, 57), and this has been shown to increase ADCC by PBMC, including NK cells and monocytes, that express activating FcγRIIIa (58–61). However, afucosylation also improves binding to FcγRIIIb ∼15 fold (44) compared to normal IgG which impacts neutrophil ADCC (2, 62), but to what extend this affects neutrophil trogoptosis toward tumor cells has not been previously investigated. Consistent with the above findings,

neutrophil-mediated ADCC of SKBR3 cells using afucosylated trastuzumab resulted in a highly significant and prominent (up to ∼80–90%) decrease in ADCC when compared to normally fucosylated trastuzumab (**Figure 4A**). Interestingly, trogocytosis was also substantially affected and showed both a decrease in the net-amount of target membrane uptake on average by neutrophils (**Figure 4B**) and decrease in the number of participating neutrophils (**Figure 4C**), confirming the negative effect of FcγRIIIb under these conditions. As expected from the above the difference in ADCC response between afucosylated and fucosylated trastuzumab became smaller when neutrophils had been activated. Furthermore, by inhibiting FcγRIIIb on neutrophils we were able to completely rescue the ability of afucosylated trastuzumab to perform ADCC and trogocytosis (**Supplementary Figure 7**) showing that the reduced killing of afucosylated trastuzumab by neutrophils can indeed be entirely attributed to its enhanced binding to FcγRIIIb. Clearly, this shows that antibody afucosylation, while enhancing the ADCC capacity of NK cells and monocytes, negatively affects neutrophil ADCC.

FcγRIIIb Contributes to IgG-Mediated Phagocytosis of Bacteria

It has previously been shown that FcγRIIIb does stimulate phagocytosis of bacteria and platelets cooperatively with other activating FcγRs, such as FcγRIIa, which is further stimulated by afucosylation of the opsonizing antibodies (7, 38, 40). To determine whether we could replicate this cooperative role we used *S. aureus* opsonized with polyclonal human IgG, which is a commercial blood product containing polyclonal IgG isolated and pooled from thousands of donors. We noticed that blocking either FcγRIIa or FcγRIIIb on neutrophils resulted in a decreased phagocytosis of *S. aureus*, with the most optimal reduction in phagocytosis being achieved by blocking both receptors (**Figure 5A**). No role for FcγRI in bacterial phagocytosis by neutrophils was found. However, since polyclonal IgG contains all IgG isotypes (approx. 65% IgG_1) and our ADCC experiments are done using only monoclonal IgG_1 antibodies we wanted to be certain that these results were not due to effects of one of the other IgG isotypes. To be able to specifically look at IgG_1 mediated effects, we used a

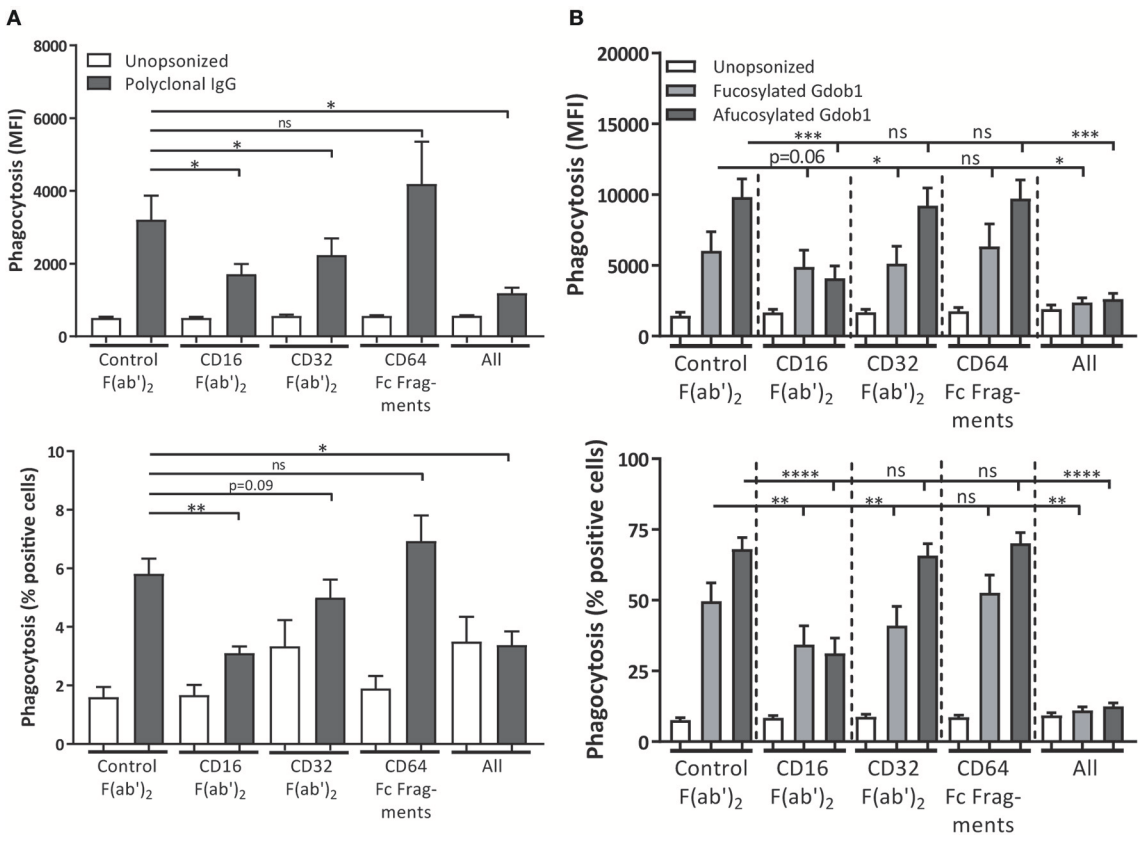

FIGURE 5 | FcγRIIa and FcγRIIIb both contribute to bacterial phagocytosis. FcγRs were blocked on freshly isolated neutrophils during phagocytosis of polyclonal IgG-opsonized *S. aureus* (gray bars) **(A)** or heat-killed *S. pneumoniae*, serogroup 6B, opsonized with GDob1 (IgG_1) (dark gray bars) or afucosylated GDob1 (IgG_1) (light gray bars) **(B)**. Shown are both percentage of neutrophils phagocytosing (% positive cells) and relative uptake of bacteria (MFI). Data shown are means + SEM with results shown from 3 **(A)**, and 2 **(B)** experiments with **(A)** $N = 10$, **(B)** $N = 7$, statistical analysis was performed by one-way paired ANOVA with Dunnett's *post-test*. ns, non-significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

heat-killed *Streptococcus pneumoniae* of serogroup 6B, which can be opsonized with a 6B-specific recombinant human IgG1 monoclonal antibody (GDob1) (47). This confirmed a cooperative role of FcγRIIa and FcγRIIIb, with the two receptors functioning in a largely redundant fashion with no additive role for FcγRI (**Figure 5B**; **Supplementary Figure 8**). Of interest, when using an afucosylated variant of GDOb1, with increased affinity for FcγRIIIb, FcγRIIIb clearly became the dominant FcγR mediating phagocytosis (**Figure 5B**). Collectively, this corroborates previous results that FcγRIIIb on human neutrophils plays a facilitating role in microbial phagocytosis, and this strongly contrasts with the negative role of this receptor during ADCC.

DISCUSSION

Here we found that FcγRIIIb on neutrophils acts as a decoy receptor during human neutrophil ADCC toward cancer cells, thereby restricting tumor killing mechanisms exerted via FcγRIIa. This is in line with previous reports showing that signaling through FcγRIIa is apparently entirely essential for active ADCC in neutrophils (2, 3). For phagocytosis, other mechanisms are apparently at play, as we and others found neutrophil FcγRIIIb to actively participate in bacterial ingestion (7, 63). This is possible as FcγRIIIb is a GPI-linked receptor, causing it to preferentially reside in detergent-resistant membranes, or lipid rafts, enriched in signaling molecules such as myristoylated src-kinases. In addition, it associates through its ectodomains with other receptors, and certainly with other FcγR in *cis* during encounter with IgG-opsonized targets, providing receptor cross-talk (4, 39, 64, 65). The enhanced recognition via FcγRIIIb apparently facilitates phagocytosis, while in contrary it impedes ADCC as we show here. Of interest, this may not only be true for phagocytosis of microbes and/or small particles, but maybe also for relatively small tumor cells such as CLL cells. Here, FcγRIIIb seems to have a beneficial effect (38, 66), although there still seems to be some discussion about whether small tumor cells are phagocytosed or in fact trogocytosed by human neutrophils (67). In general, antibodies of the IgG1 subclass bind to the various Fcγ-receptors expressed on neutrophils with a wide range of affinities. In particular, the binding affinity of FcγRIIIB for IgG1 is approximately 10-fold lower compared to FcγRIIA (68). This might explain the relative high amount of FcγRIIIB molecules on the neutrophil plasma membrane needed to create the "buffering" decoy effect of FcγRIIIB as we describe herein in the context of ADCC specifically (see **Figure 6** for a graphical representation).

In further support that FcγRIIIb negatively affects ADCC, we found a clear gene-dosage effect of *FCGR3B* through the CNV of the gene, with higher numbers gradually decreasing ADCC even further. Potentially *FCGR3B* CNV can be used as a new biomarker for cancer immunotherapy, where patients can be stratified with likelihood of benefitting from therapy when

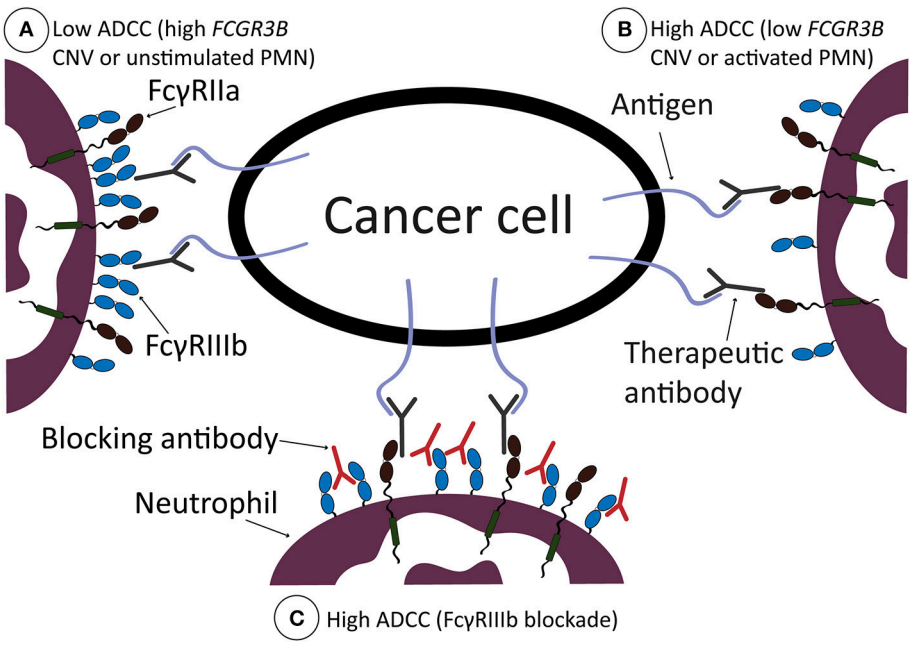

FIGURE 6 | Graphical representation of working model. ADCC by neutrophils is low when neutrophils are not stimulated, or if there is a high number of FcγRIIIb present on the neutrophil cell surface, due to high CNV of *FCGR3B* **(A)**. ADCC is high when neutrophils are stimulated, with G-CSF and IFNγ, or when there is a low number of FcγRIIIb present on the neutrophil cell surface, due to low CNV of *FCGR3B* **(B)**. ADCC by neutrophils can be increased after therapeutic intervention, i.e., by blocking FcγRIIIb with blocking antibodies, which results in high ADCC **(C)**. In all situations, FcγRIIa is required for neutrophil ADCC and is, due to high presence of FcγRIIIb in **(A)**, unable to sufficiently bind the therapeutic antibody opsonizing the cancer cell surface. When there is less FcγRIIIb present on the cell surface **(B)** or after FcγRIIIb blockade **(C)**, neutrophils are more effective in ADCC of solid cancer cells.

patients have a lower *FCGR3B* CNV combined with the right tumor antigens (e.g., HER2/Neu or EGFR).

To date, this FcγRIIIb decoy effect during ADCC has not been possible to study *in vivo* due to the fact that mice do not express a GPI-linked FcγR ortholog or homolog (27–30). However, in the future it might be interesting to study this effect in humanized models [mice expressing human FcγR or mice with human immune system (69)] to see the relative contribution of FcγRIIIb on neutrophils in therapy and if its effect can be circumvented.

Furthermore, our findings raise doubt whether the use of afucosylated monoclonal antibodies for antibody therapy against cancer is beneficial in all situations. Glycoengineering antibodies in this manner is currently being applied to various monoclonal antibodies to increase their capability to enhance ADCC and phagocytosis. This modification is well-documented to increase binding to FcγRIIIa, which is expressed by natural killer cells, monocytes and macrophages, (62, 70, 71). Less consideration has been given to the fact that this type of glycoengineering similarly enhances its affinity to FcγRIIIb, which is only present on granulocytes (44). Here, we confirm that engineered antibodies with enhanced affinity to FcγRIIIb by afucosylation have deleterious effects on ADCC by neutrophils (2, 62). This effect could partially be negated by using a combination of a targeting antibody and preventing the CD47-SIRPα- checkpoint inhibitor axis. Thus, it can be anticipated that the net effect of cancer therapeutic antibody afucosylation is basically a trade-off between the beneficial effects on various immune cells on one hand and the detrimental effects on neutrophils.

One of the obvious implications of our findings is that selective blockade of FcγRIIIb could be a potential way to enhance the effect of cancer therapeutic antibodies and thereby improve clinical outcome for patients and/or reduce their need for other non-specific agents such as chemotherapeutics. However, while interesting to explore further this is not a trivial challenge as the activating FcγRIIIa receptor on other cells has a very similar extracellular region, making it perhaps impossible to achieve the required specificity. Nevertheless, as we show here the effects of blocking FcγRIIIb appear interesting so if the issue of specificity can be solved one way or another this may be an interesting concept to pursue (see also **Figure 6** for a graphical representation).

We have shown in the current study that inhibition of FcγRIIIb also increases ADCC when this is combined with interference of CD47-SIRPα interactions. FcγRIIIb specific inhibiting agents could thus potentially be combined with antibodies targeting these checkpoint-inhibitor molecules, which are currently in development (www.clinicaltrials.gov identifiers: NCT02216409; NCT02678338, NCT02641002; NCT02367196, NCT02890368; NCT02663518, NCT02953509) (72). In theory, using a monoclonal antibody with an increased affinity to FcγRIIa (2) could also be beneficial to circumvent the decoy effect by FcγRIIIb.

Collectively, we have shown that FcγRIIIb acts as a decoy receptor for IgG during neutrophil-mediated ADCC of solid cancer cells, while it harbors a good potential to stimulate phagocytosis. These results pinpoint FcγRIIIb as a potential target and biomarker for cancer immunotherapy, while underscoring a potential threat using glycoengineered antibodies with enhanced binding to both FcγRIIIa and FcγRIIIb which needs to be further evaluated in patients.

AUTHOR CONTRIBUTIONS

LT, MvH, MH, HM, KF, RvB, TK, MvE, GV, and TvdB designed research. LT, MvH, CB, MH, XZ, JvdH, SN, PV, and JG performed research. SL-T, TV, MP, and GV contributed new reagents analytic tools. LT analyzed data. LT, HM, RvB, and TvdB wrote the paper that was edited and approved by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03124/full#supplementary-material

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MINI REVIEW
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Current Strategies to Inhibit High Affinity FcεRI-Mediated Signaling for the Treatment of Allergic Disease

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Allergies and asthma are a major cause of chronic disease whose prevalence has been on the rise. Allergic disease including seasonal rhinitis, atopic dermatitis, urticaria, anaphylaxis, and asthma, are associated with activation of tissue-resident mast cells and circulating basophils. Although these cells can be activated in different ways, allergic reactions are normally associated with the crosslinking of the high affinity Fc receptor for Immunoglobulin E, FcεRI, with multivalent antigen. Inflammatory mediators released from cytoplasmic granules, or biosynthesized *de novo*, following FcεRI crosslinking induce immediate hypersensitivity reactions, including life-threatening anaphylaxis, and contribute to prolonged inflammation leading to chronic diseases like asthma. Thus, inappropriate or unregulated activation of mast cells and basophils through antigenic crosslinking of FcεRI can have deleterious, sometimes deadly, consequences. Accordingly, FcεRI has emerged as a viable target for the development of biologics that act to inhibit or attenuate the activation of mast cells and basophils. At the forefront of these strategies are (1) Anti-IgE monoclonal antibody, namely omalizumab, which has the secondary effect of reducing FcεRI surface expression, (2) Designed Ankyrin Repeat Proteins (DARPins), which take advantage of the most common structural motifs in nature involved in protein-protein interactions, to inhibit FcεRI-IgE interactions, and (3) Fusion proteins to co-aggregate FcεRI with the inhibitory FcγRIIb. This review presents the published research studies that support omalizumab, DARPins, and fusion proteins as, arguably, the three most currently viable strategies for inhibiting the expression and activation of the high affinity FcεRI on mast cells and basophils.

Keywords: FcεRI, allergy, omalizumab, DARPin, fusion protein, mast cells, basophils, FcγRIIb

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INTRODUCTION

Allergic disease refers to a variety of disorders that include seasonal allergies, atopic dermatitis, urticaria, life-threatening anaphylaxis reactions to food, and allergic asthma. Curiously, the incidence of allergic disease has increased dramatically in recent decades, and continues to rise in developed countries. Allergies and asthma are among the most prevalent chronic diseases worldwide (1, 2). The culprits are a variety of pre-formed inflammatory mediators including histamine, serine proteases, proteoglycans, and other enzymes, that are stored in cytoplasmic granules and released from mast cells and basophils immediately following "degranulation," and eicosanoids like prostaglandins and leukotrienes that are very rapidly biosynthesized from

arachidonic acid. Prolonged stimulation also induces the activation of various transcription factors, and synthesis of new cytokines that contribute to inflammation and recruitment of other cell types.

Mast cells can be activated by a variety of agents. However, allergic reactions are generally associated with crosslinking of the high affinity Fc receptor for immunoglobulin E (IgE), FcεRI, with multivalent antigen (3). High affinity FcεRI is comprised of an IgE-binding α chain, a signal enhancing β chain, and two signal transducing γ chains. The tetrameric receptor, αβγ2, is expressed predominantly on tissue-resident mast cells and circulating basophils (4). However, in a proportion of human subjects, mostly atopic patients, a trimeric form of the receptor lacking the β chain, αγ2, is expressed on other cell types including airway smooth muscle (5), bronchial and intestinal epithelial cells (6, 7), Langerhan cells (8, 9), dendritic cells (10, 11), monocytes (12), and eosinophils (13), neutrophils and platelets (14–16).

Binding of IgE to FcεRI on mast cells and basophils enhances FcεRI expression (17–21). It is thought that IgE binding to FcεRI protects the receptor from being internalized and degraded. On the other hand, IgE binding to FcεRI on dendritic cells and monocytes (but not basophils) facilitates the internalization and degradation of IgE-bound FcεRI within endolysosomal compartments (22). In addition to showing that IgE levels are important in stabilizing FcεRI expression, these observations also indicate a role for FcεRI in clearance of serum IgE. Moreover, they suggest that αβγ2 expressed on mast cells and basophils is predominantly involved in signal transduction leading to mast cell and basophil activation or degranulation, whereas αγ2 on antigen presenting cells is mostly involved in IgE-FcεRI internalization.

The role of FcεRI as the primary activator of mast cells and basophils leading to the release of allergic/inflammatory mediators resulting in IgE-mediated immediate hypersensitivity reactions and allergic inflammation is well-documented (3). Accordingly, FcεRI has emerged as a target of biologics for regulating allergic reactions. Currently, anti-IgE monoclonal antibody omalizumab, DARPins, and fusion proteins that co-aggregate FcεRI and FcγRIIb are at the forefront of the strategies currently employed or actively being investigated as a means of regulating the expression and/or activation of FcεRI for the therapeutic purpose of inhibiting mast cells and basophils (**Figure 1**).

OMALIZUMAB

Perhaps the most studied strategy directed against allergic disease is the use of anti-IgE antibodies. Omalizumab (Xolair®) is a humanized anti-IgE mouse monoclonal antibody that is FDA-approved for the treatment of mild to severe allergic asthma and chronic spontaneous urticaria (23–26). Omalizumab works by binding to circulating free IgE, thereby, reducing the amount that would normally be available to bind FcεRI on mast cells and basophils. In an early Phase I study of 15 allergic and asthmatic patients with serum levels of IgE between 187 and 1,210 ng/ml, intravenous injection of omalizumab resulted in reduction of IgE to 1% of pre-treatment levels (27). It is widely reported that omalizumab competes with FcεRI for the C3ε domain of IgE, thus preventing it from binding FcεRI-bound IgE (28, 29). However, another study reported that steric hindrance by C2ε domain, rather than direct competition for site binding, was responsible for the inability of omalizumab to bind FcεRI-bound IgE (30). Regardless, omalizumab cannot bind IgE bound to FcεRI on mast cells or basophils, and, therefore, does not crosslink IgE-bound FcεRI to induce the release of allergic mediators. Since binding of IgE to FcεRI on mast cells and basophils enhances the expression of FcεRI (17–21), the reduction in free IgE by omalizumab leads to diminished expression of FcεRI on the surface of mast cells, basophils, and dendritic cells (21, 27, 31, 32). In one study, treatment of atopic individuals with omalizumab for 3 months reduced the expression of FcεRI on basophils by ∼97% from ∼220,000 to ∼8,300 receptors per basophil (27). An *in vitro* study with *in situ*-matured mast cells from human skin demonstrated that IgE-dependent enhancement of FcεRI on human skin mast cells was both prevented and reversed by omalizumab (21). In this study, omalizumab prevented the upregulation of FcεRI by 90% when added simultaneously with polyclonal IgE at a molar ratio of 2.9 (omalizumab to IgE). Omalizumab also dose-dependently decreased FcεRI expression on human skin mast cells when added to cultures after FcεRI had already been upregulated with IgE, suggesting that omalizumab could disassemble pre-formed IgE:FcεRI complexes. This was later confirmed with a cell-free system and human basophils (30, 33). The exact mechanism by which omalizumab "strips" IgE off of FcεRI is not exactly known, but allosteric destabilization and facilitated dissociation of the IgE:FcεRI complex, at least at high concentrations of omalizumab, are suspected (33–36). Human skin mast cells with IgE-enhanced FcεRI levels were more sensitive to stimulation with a low dose of anti-FcεRI mAb compared to mast cells with basal levels of FcεRI in terms of degranulation, PGD_2 biosynthesis, and cytokine production. Reduction of FcεRI levels with omalizumab restored sensitivity to stimulation, and mediator release, to basal levels.

The efficacy and safety of omalizumab as treatment against allergic asthma and urticaria has clearly been demonstrated, including as an add-on therapy with traditional treatments such as glucocorticoids (23, 24). The therapeutic potential of omalizumab in other IgE-mediated disorders in which FcεRI plays a role, including food allergy (37–39), allergic rhinitis (40, 41), and atopic dermatitis (42, 43) has also been demonstrated. However, one major concern is the duration of the positive effects of omalizumab post-treatment. In one study (44), serum free IgE was reduced by 96–98%, and wheal-and-flare reactions to skin prick tests were significantly reduced in 40 patients with allergic rhinitis who were treated with omalizumb for 28 weeks. However, serum free IgE levels and skin reactivity increased following a reduction in the amount of omalizumab administered, and returned to baseline when therapy was completely discontinued. In another study (45), loss of control of asthma symptoms following discontinuation of omalizumab was recorded in 57% of the participants with a median time-point of 13 months after discontinuation. In these studies, FcεRI levels on mast cells or basophils was not monitored, but

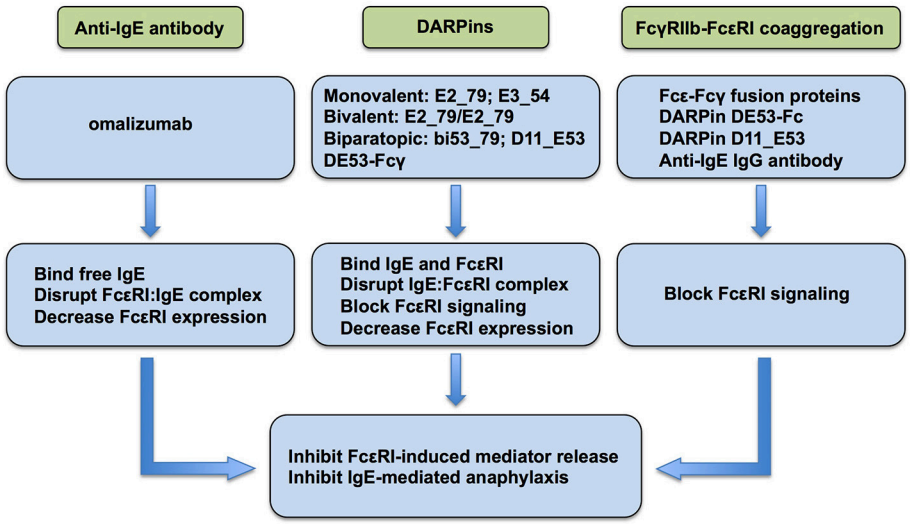

FIGURE 1 | Current strategies to inhibit FcεRI signaling in allergic disease.

given that omalizumab decreases FcεRI expression on these cell types (21, 27, 31, 32), it is expected that receptor expression increased when treatment was terminated. Thus, treatment with omalizumab could require personalized optimization in terms of dosage and duration of treatment to yield maximal benefits.

Omalizumab as an adjunct to allergen immunotherapy (AIT) against IgE-mediated food allergy and allergic asthma is also currently under investigation (46–50). The main types of AIT are subcutaneous immunotherapy (SCIT) and sublinguinal immunotherapy (SLIT) (51). SCIT and SLIT have been shown to be efficacious for perennial and seasonal allergic respiratory disease (50, 52, 53). However, SCIT or SLIT are contraindicated for severe or uncontrolled asthma (54). It is thought that pre-treatment with omalizumab of patients with severe uncontrolled asthma, which has been shown to be efficacious, could allow AIT in patients that previously could not tolerate it (48, 55). However, studies to investigate AIT in combination with omalizumab are currently lacking. With regard to food allergies, omalizumab treatment in conjunction with oral immunotherapy (OIT) has shown promise in desensitizing allergic patients to peanuts, milk, and multiple food allergens (56–60). Overall, the few reported studies have shown promise for the use of omalizumab in combination with AIT for IgE-mediated disease.

Other anti-IgE antibodies have also been developed and tested including Ligelizumab (QGE031), Quilizumab (MEMP1972A), XmAb7195, and MEDI4212 that might provide additional opportunities for anti-IgE therapy in the future (61). To date, however, none have been shown to be clinically superior to omalizumab, or data is still coming out. In some cases, for example QGE031 for asthma, development has been discontinued. Nevertheless, these or other anti-IgE antibodies could provide additional opportunities for anti-IgE therapy in the future.

DARPINS

DARPins (designed ankyrin repeat proteins) are a class of small (14–21 kDa) binding proteins comprised of a varying number of stacked ankyrin repeat domains (62), which are one of the most common structural motifs involved in protein-protein interactions in nature. Natural ankyrin repeats are 33 residue motifs comprised of two α-helical structures connected by a loop that stack one on top of the other to form ankyrin repeat domains (63). A single DARPin library module is comprised of a 33 residue repeat of which seven residues are randomized and non-conserved. Typically, two to four library modules are genetically fused and flanked by N-cap and C-cap repeats to form one protein domain (64, 65). Binding of ankyrin repeat domains can affect stability and effector function of the target protein. The motivation for engineering DARPins was to generate binding proteins that could be used to target proteins with high affinity and specificity, essentially replacing the use of monoclonal antibodies (62).

In one of the first studies (66), two monovalent DARPins (B-A4-85 and C-A3-30) capable of binding two different epitopes of human FcεRIα were identified and successfully fused to each other with the flexible linker $[Gly_4\text{-}Ser]_4$. A bispecific DARPin (30/85) was identified as being capable of simultaneously binding FcεRIα at both epitopes with affinity for FcεRIα greater than that of IgE. In *in vitro* studies, DARPin 30/85 blocked IgE binding to FcεRI, and inhibited IgE-induced degranulation of human FcεRIα-transfected RBL-2H3 cells to a similar extent as omalizumab. In a similar study (67), two monovalent DARPins, E2_79 and E3_54, that were specific for IgE, and could inhibit IgE-FcεRI interactions, were identified. Bivalent proteins were genetically engineered by coupling the monovalent DARPins with the glycine-serine linker. E2_79/E2_79, at 5-fold molar excess with IgE, inhibited the binding of IgE to FcεRIα by >90%, comparable binding

by omalizumab. E2_79/E2_79 also effectively bound free IgE in serum. The researchers further demonstrated that both the monovalent and bivalent DARPins inhibited IgE-mediated degranulation of FcεRIα-transfected RBL-2H3 cells. Bivalent DARPin E2_79/E2_79 was particularly effective, exhibiting an IC_{50} of 0.54 nM compared to 1.77 nM for omalizumab. It was later shown that E2_79, in addition to binding free IgE, could also stimulate the dissociation of pre-formed IgE:FcεRI complexes by a facilitated dissociation mechanism at one of two binding sites identified for E2_79 on the IgE:FcεRI complex (36). In a separate study, treatment with E2_79 significantly reduced surface expression of FcεRI on human *ex vivo* isolated primary basophils, and inhibited FcεRI-induced activation and leukotriene C4 (LTC_4) biosynthesis (30). Further, a biparatopic DARPin, bi53_79, which was engineered by fusing the disruptive E2_79 with non-disruptive E3_53 anti-IgE DARPins exhibited a >10-fold increase in capacity to disrupt FcεRI:IgE complexes, and was more effective at inhibiting anaphylactic reactions *in vivo* compared with E3_79 alone. Noteworthy, E2_79 and bi53_79 acted faster and were more effective than omalizumab in parallel experiments. These studies demonstrate the therapeutic potential of DARPins as inhibitors of FcεRI-induced allergic reactions. Thus, supporting the notion that DARPins have the potential to supplant monoclonal antibodies such as omalizumab as treatment for allergic asthma and other allergic diseases (62, 65).

However, DARPins are protein structures, and the potential for immunoreactivity resulting from the production of anti-DARPin antibodies should be met with extreme caution. Clearly the immune response to DARPin proteins could be a major limitation in the use of DARPins as therapeutic agents. In addition, the possibility of negative or deleterious effects of inhibiting the activation of FcεRI-expressing cell types should also be considered. For example, mast cells and eosinophils play a major role in the clearance and expulsion of parasites particularly helminths. Likewise, mast cell mediators also protect against insect and reptile venom. Thus, blocking the activation of mast cells could inhibit the positive or protective effects associated with FcεRI activation. This might be particularly relevant in countries where parasitic infections are endemic. It is argued that DARPins would be more cost effective than monoclonal antibodies because they can be produced in large scale in bacteria; however, the relative cost to human safety must be considered. Importantly, in July 2018, Allergan and Molecular Partners announced that Abicipar pegol, a DARPin engineered to target vascular endothelial growth factor (VEGF), had reached the primary end point in two Phase III trials for the treatment of neovascular age-related macular degeneration (AMD). In two trials, Abicipar pegol demonstrated non-inferiority to the approved anti-VEGF ranibizumab (Lucentis®). Of significant concern, however, was a significantly greater incidence of ocular inflammation with Abicipar pegol than Lucentis®. Allergan is expected to file Abicipar pegol with the FDA in early 2019. Thus, whether DARPins are safe and efficacious in humans is currently being determined.

CO-AGGREGATION OF FCεRI WITH FCγRIIB

Given the requirement for tyrosine phosphorylation events in the initiation and propagation of FcεRI signaling in mast cells and basophils (68–72), one strategy to inhibit FcεRI-mediated reactions has been to take advantage of the inhibitory property of FcγRIIb. FcγRIIb is the only known inhibitory IgG Fc receptor (73, 74). In contrast to FcεRI, which utilizes immunoreceptor tyrosine-based activation motif (ITAM), FcγRIIb utilizes the inhibitory counterpart (ITIM) that, upon receptor activation, recruits SH2-domain containing phosphatases including SHIP. The phosphatases interfere with the tyrosine-based activation of early signaling molecules resulting in the inhibition of signal transduction (75–77). FcγRIIb is expressed on human basophils and cord blood-derived mast cells (78–80). It is not constitutively expressed on human skin mast cells (81), but FcγRIIb expression can be induced in human intestinal mast cells with interferon γ (82) and on human basophils with IL-3 (79) suggesting that it could be induced in tissue-derived mast cells. Various experiments have been performed demonstrating that co-aggregation of FcεRI and FcγRIIb inhibits IgE-dependent activation and mediator release from mast cells and basophils. In one study (83), it was demonstrated that serotonin release from mouse bone marrow-derived mast cells (BMMCs) sensitized with anti-ova IgE, and then challenged with ova, was dose-dependently inhibited when the BMMCs were challenged with DNP-ova complexed with anti-DNP IgG. The requirement for co-aggregation of FcεRI and FcγRIIb to inhibit mast cell mediator release was further tested and confirmed in rat basophilic leukemia cells (RBL-2H3) transfected with FcγRIIb. Another study (84) used a bispecific antibody expressing one Fab fragment specific for human IgE, and the other for FcγRIIb, to show that simultaneous crosslinking of FcεRI and FcγRIIb inhibited antigen induced histamine release from human cord blood-derived mast cells and peripheral blood basophils. Cassard et al. (79) used an IgG anti-IgE, which binds FcεRI-bound IgE via its Fab, and FcγR via their Fc domain, to demonstrate that co-aggregation of FcεRI and FcγRIIb negatively regulates IgE-induced activation of human and mouse basophils, and release of histamine and IL-4. Furthermore, a comprehensive *in vivo* study utilizing passive and active immunization of mice determined that FcεRI-FcγRIIb crosslinking contributed significantly to the inhibition of IgE-mediated anaphylaxis by IgG blocking antibodies particularly under low concentrations of IgG blocking antibody (85). Collectively, these studies support the notion that co-aggregation of FcεRI and FcεRIIb is a viable strategy to limit allergic responses.

Over the years, Fcε-Fcγ fusion proteins to co-aggregate FcεRI and FcγRIIb have been investigated. One of the earliest bi-functional fusion proteins that was engineered, termed GE2, is comprised of the hinge-Cγ2-Cγ3 domains of the human IgG Fc and Cε2-Cε4 domains of human IgE Fc connected by a 15 amino acid $(Gly_4\text{-}Ser)_3$ linker (86). Human GE2 was shown to bind to both FcεRI and FcγRII at levels equivalent to human IgE and IgG, respectively. Functionally, GE2 inhibited

IgE-dependent degranulation of human basophils in time- and dose-dependent manner with maximal inhibition observed when the cells were sensitized with antigen-specific IgE and GE2 simultaneously. GE2 co-aggregation of FcεRI and FcγRII inhibited Syk phosphorylation, a critical event in FcεRI signaling (87, 88), and *in vivo* IgE-induced passive cutaneous anaphylaxis in transgenic mice expressing a human FcεRIα. Kepley, et al. (78) subsequently used GE2 to further demonstrate that co-aggregation of FcεRI and FcγRII on human umbilical cord blood-derived mast cells inhibited degranulation and cytokine production. In a similar study, Mertsching et al. (89) created a murine homolog of human GE2, termed mGE, consisting of $C\gamma_{2a}2$-$C\gamma_{2a}3$ and Cε2-Cε3-Cε4 domains connected by the $(Gly_4\text{-}Ser)_3$ linker. mGE was shown to inhibit IgE-dependent degranulation and cytokine production from wild type but not FcγRIIb-deficient mice BMMCs. mGE also inhibited *in vivo* passive cutaneous and systemic anaphylaxis in mice, with extended protection. Conversely, mGE treatment increased FcγRIIb phosphorylation and its association with SHIP and SHP1/2 phosphatases.

In an effort to enhance the efficacy of FcεRI-FcγRIIb co-engagement while eliminating the possibility of FcεRI crosslinking, Cemerski et al. (90) engineered a tandem Fcε-Fcγ fusion protein comprised of a murine Fcε domain linked to a human Fcγ domain IgG_1, which, due to S267E and L328F amino acid substitutions at the Fcγ domain, exhibited >100-fold greater affinity for human FcγRIIb compared to the native IgG Fc composition (91, 92). This fusion protein was shown to inhibit IgE-dependent degranulation of human FcγRIIb transgenic BMMCs. However, in the reported experiments, the tandem fusion protein containing the native IgG Fc domain inhibited mast cell degranulation to a similar extent as a control tandem fusion protein lacking affinity for FcγRIIb. The authors concluded that inhibition of mast cell degranulation by co-engagement is more potently suppressed when the tandem fusion protein has higher affinity for FcγRIIb. To our knowledge, the tandem Fc fusion protein with enhanced affinity for FcγRIIb has not been compared to the other reported FcεRI-FcγRII fusion proteins, GE2 (86) and hGE2 (89).

Two pre-clinical studies in non-human primates have demonstrated the potential clinical applicability of FcεRI-FcγRIIb fusion proteins in inhibiting allergic reactions. Zhang et al. (93) first demonstrated that GE2 could inhibit mediator release from mast cells and basophils that had been pre-sensitized with IgE before treatment with GE2 as would be the case in allergic individuals undergoing treatment. The researchers demonstrated that GE2 inhibited Fel d 1 (cat allergen)-induced histamine release from human basophils and lung mast cells from cat allergic patients. Mirroring this, GE2 blocked Fel d 1-induced passive cutaneous anaphylaxis in human FcεRIα transgenic mice that were sensitized with serum from cat allergic subjects. GE2 itself was shown to not induce mediator release or induce anaphylaxis. In their pre-clinical study, GE2 was shown to inhibit skin test reactivity to dust mite (*Dermatophagoides farinae*) allergen in Rhesus monkeys that were naturally allergic to the *D. farina* allergen. In a later study, Mertsching et al. (89) generated another FcεRI-FcγRIIb fusion protein, termed hGE2, based on the GE2 construct of Zhu et al. (86) absent of any non-native sequences. hGE2, administered to cynomolgus monkeys that had been sensitized with the roundworm *Ascaris suum*, was shown to protect the monkeys from cutaneous anaphylaxis induced with *A. suum* extract. The monkeys were reportedly protected from local anaphylaxis for up to three weeks.

Interestingly, a humanized monoclonal anti-IgE antibody (XmAb7195) was reported to have an IgE-binding affinity 5.3-fold greater than omalizumab, and 400 times greater binding affinity for FcγRIIb due to mutations in its Fc region (94). XmAb7195 was shown to block free IgE and inhibit IgE production in B cells by co-engaging IgE and FcγRIIb. Although XmAb7195 did not bind FcεRI-bound IgE (94), this study supports the notion of using anti-IgE IgG antibodies to co-aggregate FcγRIIb and FcεRI to inhibit allergic disease. First-in-Human Phase 1 clinical trials have been conducted with XmAb7195, but results on safety, tolerability and bioavailability have not been reported (61).

DARPins have also been used to co-aggregate FcεRI and FcγRIIb. Eggel et al. (95) generated an anti-IgE DARPin fusion protein in which DARPin E53, which showed reactivity against a non-FcεRIα epitope capable of binding free and receptor-bound IgE, was joined via the $(Gly_4\text{-}Ser)_3$ linker to a human IgG_1 Fc region. DE53-Fc, as it was named, was shown to not be anaphylactogenic, and inhibited allergen-induced activation of basophils in whole blood samples from allergic donors. In a subsequent study (96), a DE53-Fc mutant construct with increased affinity for FcγRIIb due to a single site-directed point mutation in the IgG Fc region was shown to be more efficient at co-aggregating FcεRI and FcγRIIb, resulting in enhanced inhibition of basophil activation. Recently, Zellweger et al. (97) generated DARPin D11_E53, which simultaneously bound human FcγRIIb and FcεRI-bound IgE. The bispecific molecule was shown to inhibit allergen-induced degranulation and LTC_4 biosynthesis in human primary basophils and huFcεRIα-expressing mouse BMMCs *in vitro*, and decreased *in vivo* passive systemic anaphylaxis induced in huFcεRIα transgenic mice. This study demonstrated that FcγRIIb-mediated inhibition of degranulation requires direct ligation with FcεRI, and that DARPins, at least D11_E53, could safely be applied to animals to inhibit anaphylaxis.

CONCLUDING COMMENTS

The dramatic increase in prevalence of allergies warrants additional research to develop new strategies and therapies to treat allergic disease. At the forefront are the anti-IgE monoclonal antibody omalizumab, DARPins, and fusion proteins that directly or indirectly alter FcεRI expression and activation. In order to maximize the use of omalizumab, additional clinical studies are needed to identify allergic diseases against which omalizumab could be effective beyond asthma and spontaneous urticaria. The development of newer anti-IgE antibodies could also have an impact. The development of DARPins hold the promise of targeting FcεRI or IgE with greater specificity and better efficacy than monoclonal antibodies without the

hurdles associated with development of humanized monoclonal antibodies. As potential clinical therapeutics, DARPins also have the potential to reach a broader population since allotypic differences associated with the use of monoclonal antibodies might not factor in their development. However, safety issues regarding immunogenicity due to anti-DARPin antibodies and unwanted effects due to inhibiting positive effects of mast cell activation must be considered. Whether DARPins can supersede monoclonal antibodies remains to be determined. Harnessing the inhibitory properties of FcγRIIb to inhibit FcεRI with fusion proteins also shows promise as evidenced in pre-clinical studies with non-human primates. It is hoped that these strategies will lead to therapeutics that provide relief to the millions of people worldwide suffering from allergic disease.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Multiple Variables at the Leukocyte Cell Surface Impact Fc γ Receptor-Dependent Mechanisms

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Fc γ receptors (FcγR) expressed on the surface of human leukocytes bind clusters of immunoglobulin G (IgG) to induce a variety of responses. Many therapeutic antibodies and vaccine-elicited antibodies prevent or treat infectious diseases, cancers and autoimmune disorders by binding FcγRs, thus there is a need to fully define the variables that impact antibody-induced mechanisms to properly evaluate candidate therapies and design new intervention strategies. A multitude of factors influence the IgG-FcγR interaction; one well-described factor is the differential affinity of the six distinct FcγRs for the four human IgG subclasses. However, there are several other recently described factors that may prove more relevant for disease treatment. This review covers recent reports of several aspects found at the leukocyte membrane or outside the cell that contribute to the cell-based response to antibody-coated targets. One major focus is recent reports covering post-translational modification of the FcγRs, including asparagine-linked glycosylation. This review also covers the organization of FcγRs at the cell surface, and properties of the immune complex. Recent technical advances provide high-resolution measurements of these often-overlooked variables in leukocyte function and immune system activation.

Keywords: antibody, IgG, N-glycosylation, post-translation modification, ADCC—antibody dependent cellular cytotoxicity, immune complex, ADCP—antibody dependent cellular phagocytosis

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INTRODUCTION: THE IMPORTANCE OF MODULATING THE Fc-FcγR INTERACTION

Immunoglobulin G (IgG) is the most thoroughly studied and well characterized molecule of the humoral immune response. IgG activates the immune system through cell-bound Fc γ Receptors (FcγRs; **Figure 1**). The IgG fragment antigen-binding (Fab) domains confer specificity and affinity toward an antigen while the distinct hinge and fragment crystallizable (Fc) domain of the four IgG subclasses (IgG1-4) provide the structural basis for specificity and affinity to bind FcγRs (1). The six structurally distinct members of the classical human FcγRs (FcγRI or CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, FcγRIIc/CD32c, FcγRIIIa/CD16a, and FcγRIIIb/CD16b) are expressed on leukocytes of both the myeloid and lymphoid lineage (**Figure 2**). This group of proteins can be divided into two types: activating receptors (CD64, CD32a, CD32c, CD16a, and CD16b) that lead to cell activation through immunoreceptor tyrosine-based activation motifs (ITAM) on cytosolic tails or on co-receptor molecules, and an inhibitory receptor (CD32b) that signals through immunoreceptor tyrosine-based inhibitory motifs (ITIM) (2–4). Only CD32s contain ITAM or ITIM domains, and the other receptors must associate with an ITAM-containing

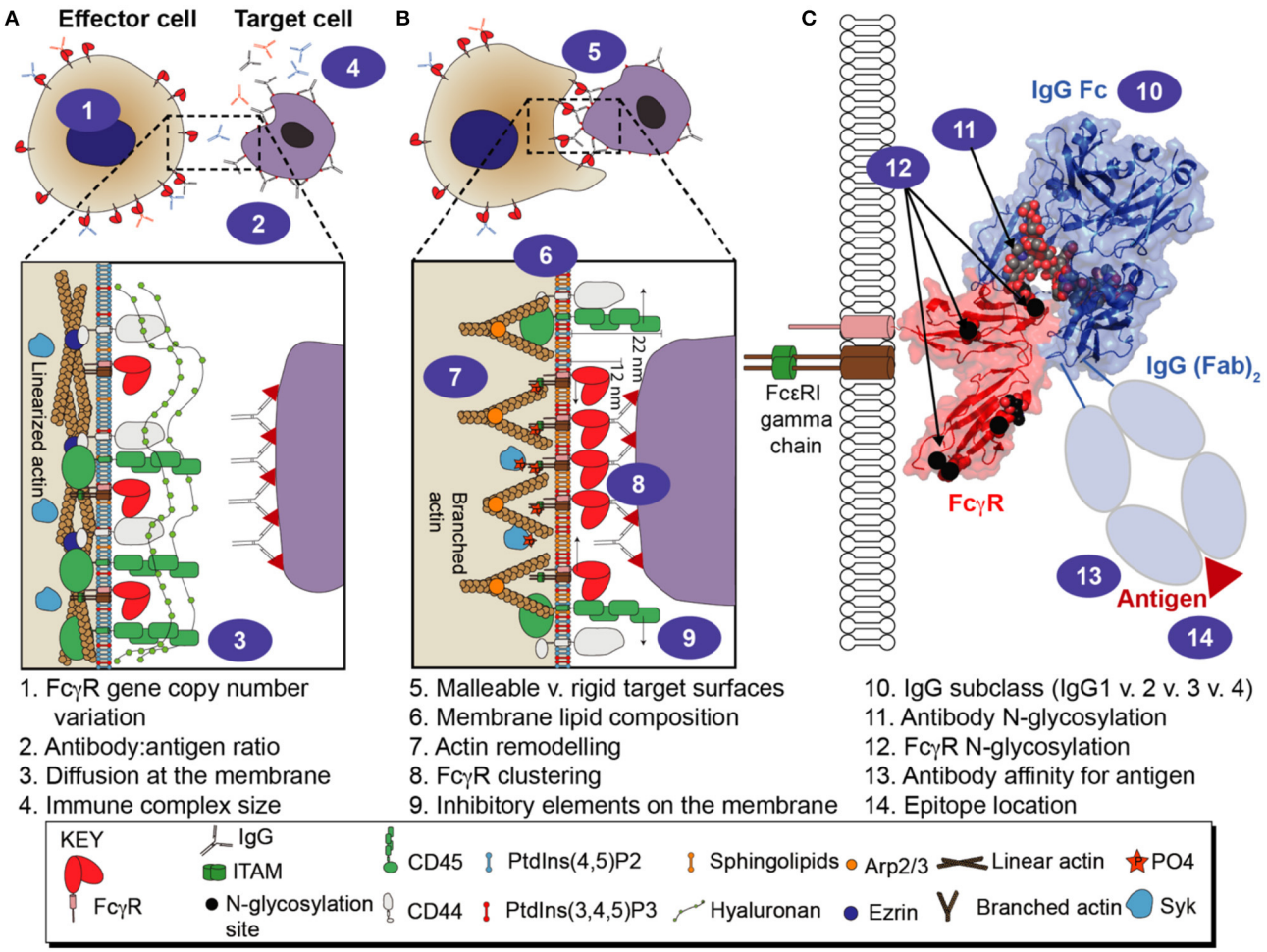


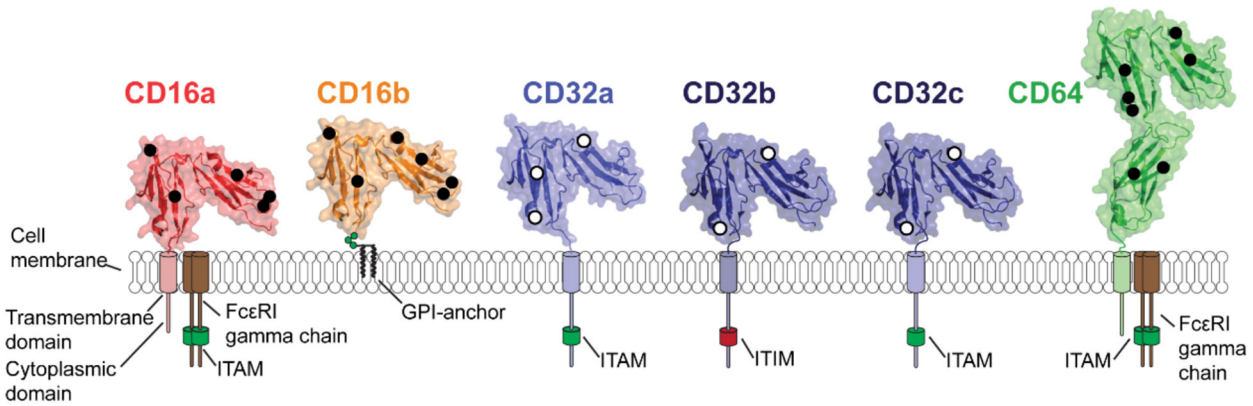
FIGURE 1 | Multiple variables affect FcγR-mediated immune function. **(A)** cellular variables influencing FcγR activity that are present before the effector cell engages a target cell. **(B)** cellular variables influencing FcγR-mediated activity while the effector cell is engaged with a target cell. **(C)** molecular variables associated with the FcγRs, antibody, and antigen.

adaptor protein (FcεRI γ chain or CD3 ζ chain) (3, 5) (**Figure 2**). In either situation, the ratio of activating to inhibiting signals determines the outcome of an immune response (6).

Receptor clustering is essential for FcγR signaling. Circulating IgG coats an antigen to form an oligomeric complex, positioning the Fc portions of the IgG molecules away from the target surface and exposed to interact with FcγRs. The antibody-coated target is also referred to as an immune complex. The multiple IgG molecules of the immune complex provide an opportunity for multivalent interactions with FcγR-expressing leukocytes and must compete with non-complexed serum antibodies occupying the FcγRs that will, in turn, cluster FcγRs on the cell surface (7, 8). Depending on the receptors engaged, the clustering of the extracellular domains triggers phosphorylation of tyrosine in the ITAMs or ITIMs, which subsequently recruits signaling molecules that promote a cellular response (9). The types of FcγR-mediated effector cell responses are diverse and include, but are not limited to, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), release of cytokines and antigen uptake for presentation (10–14). FcγRs are critical for maintaining immune system homeostasis as well as preventing pathogenic infections and they play a major role in inflammatory diseases and autoimmune disorders (9, 13, 15–17). The combination of distinct antagonistic and synergistic factors contribute to a considerable functional diversity within this group of antibody receptors. Here we will discuss multiple factors which influence the antibody:FcγR interaction and modify the immune response (**Figure 1**).

RECEPTOR PRESENTATION AT THE CELL SURFACE

FcγRs are predominately expressed on cells originating from hematopoietic progenitor stem cells including dendritic cells, neutrophils, basophils, eosinophils, macrophages, monocytes,



N-glycan sites	5	4 or 6	3	2	2	7
Cellular function	Activation	Activation	Activation	Inhibition	Activation	Activation
Affinity to ligand	IgG3>IgG1> IgG4=IgG2	IgG3>IgG1	IgG1>IgG3> IgG2=IgG4	IgG1=IgG3= IgG4>IgG2	IgG1=IgG3= IgG4>IgG2	IgG1=IgG3 >IgG4
Expression in human hematopoietic cells	NK cell Monocyte* Macrophage* CD4+ T cell* Basophil* Mast cell*	Neutrophil Basophil* Eosinophil**	Macrophage Neutrophil Platelet Mast cell Eosinoplil	B cell Macrophage Dendritic cell Basophil Monocyte* Neutrophil*	B cell*** NK cell***	Macrophage Monocyte Dendritic cell Mast cell Neutrophil**

* expression in a subset
** induced expression
*** expressed in a subset of cells

FIGURE 2 | Structures and properties of the human Fc γ receptors. Five receptors are expressed in the majority of the population with CD32c expressed only in a small subset. The ribbon diagrams show the structures of the extracellular antibody-binding domains as determined by x-ray crystallography; overlayed black or white circles indicate the sites of N-glycosylation.

mast cells, NK cells, B cells, a subset of T cells, and platelets as well as non-hematopoietic cell types such as syncytiotrophoblasts at various levels (18–20). FcγR expression varies depending on cell lineage; not surprisingly gene copy number is also implicated in disease. These factors can greatly influence the dynamic ability of the immune system to respond to the diverse repertoire of foreign invaders. Thus, variable surface expression by different immune cell types influences how the immune system responds to a foreign invader. This section will cover the cellular expression of FcγRs and immune modulation of expression through downregulation and induction.

Five activating FcγRs are expressed in humans (**Figure 2**). The highest affinity, CD64, is expressed on monocytes, dendritic cells and macrophages (11), mast cells (21), and neutrophils following IFN-γ exposure (22, 23). The low affinity CD32a is expressed on mast cells, neutrophils, macrophages, eosinophils, and platelets (24). CD32c is expressed by 7–15% of individuals on NK cells and B cells and results from a gene mutation (4). The high/moderate affinity CD16a is expressed predominantly on NK cells, a subset of monocytes, mast cells, basophils, macrophages and is inducible in CD4+ T-cells (25, 26). The low/moderate affinity CD16b is found only in humans and expressed predominantly on neutrophils (27), a subset of basophils (28) and has inducible expression on eosinophils (29, 30). CD32b is the sole inhibitory receptor and is expressed on basophils, B cells, macrophages, dendritic cells, a subset of monocytes and neutrophils (24). Interestingly, CD32b is also expressed in non-hematopoeitic cells, including the endothelium of various organs (31).

Variability in Receptor Amount

Gene duplications in individuals lead to copy number variation (CNV) of some FcγRs in the population. Surprisingly, only CD16a, CD16b, and CD32c of the FcγRs exhibited CNV in a sample population of 600 subjects (32). CNVs have been correlated to autoimmune disorders as well as variations in surface expression levels. CNV of CD16b is correlated to surface expression on neutrophils and implicated in SLE susceptibility (33, 34), as well as other autoimmune disorders (35, 36). Furthermore, CD16a CNV appears to be functionally significant since increased surface expression positively correlated with increasing CD16a gene number (ranging from one to three copies) (32, 35). A CD16a indel has been shown to increase surface expression as well (37).

FcγR amount at the cell surface varies by cell type and receptor identity (**Figure 2**). On neutrophils, there are an estimated 100,000–300,000 surface exposed CD16b molecules and 10,000–40,000 CD32a molecules (38, 39). The predominant monocyte subtype at roughly 80% of the pool, "classical" monocytes, does not express CD16a. "Non-classical" monocytes express CD16a at a level of roughly 10,000 CD16a molecules per cell but upon differentiation into macrophages express 40,000 CD16a molecules per cell while CD32 remained the same at ∼10,000 molecules per cell (40). Another study found macrophages express 5–10 fold higher CD64, CD32a, and CD32b while CD16a expression was comparable to non-classical monocytes. M2c macrophages expressed overall higher levels of FcγRs than M1 macrophages with the following order of expression: CD32a, CD32b > CD64 > CD16a (41). A high number of CD16a molecules are expressed on CD16+ NK cells (100,000-250,000) (42).

Expression levels also vary based on the cell status. Following activation, innate immune cells can induce expression of FcγRs (23, 25, 29, 30, 35). There is also evidence of receptor downregulation upon activation. Downregulation mechanisms include both decreases in expression as well as shedding FcγR from the cell surface following metalloproteinase cleavage. CD32a is shed from Langerhans cells and also expressed as a soluble form (43). CD32b is shed upon activation of B-cells (44). CD16a and CD16b are likewise shed upon activation of NK cells and neutrophils at a known cleavage site by the metalloprotease ADAM17 (45–48). Intriguingly, sCD16b is relatively abundant in serum (∼5 nM) (49) and levels vary based on the immune state of the individual (50). Surprisingly, CD64 is the only human FcγR in which a soluble, serum-borne form has not been reported. This may be explained by the presence of a third extracellular CD64 domain in place of the cleavage site found in CD32s and CD16s (**Figure 2**).

Soluble FcγR forms modulate immune responses. Soluble CD16b binds myeloid cells, NK cells, subsets of T cells, B cells, and monocytes through complement receptor 3 (CR3 or Mac-1 or αM β2, comprised of CD11b/CD18) and complement receptor 4 (CR4 or αx β2, comprised of CD11c/CD18). These interactions cause the release of IL6 and IL8 by monocytes and indicate a potential role for soluble CD16b in inflammation (51). Shedding of CD16a from NK cells allows disengagement of the immune synapse from the target cell and the subsequent ability to kill again. One study demonstrated that repeated engagement by CD16a depleted perforin, however, shedding of CD16a allowed perforin replenishment upon subsequent activation by another activating receptor, Natural killer group 2 member D (NKG2D), which recognizes ligands not normally expressed on healthy tissue (52). Thus, it appears that the act of shedding of CD16 can allow disengagement of the foreign particle which would be crucial for the immune cell's survival and preservation of potential future cytolytic activity. Though shed receptors are proinflammatory and recruit immune cells as discussed above, a complete picture of the mechanisms of regulating surface expression upon immune activation is not currently available.

Receptor Clustering at the Membrane Is Required for Effector Function

The correct presentation of FcγRs on the cell membrane is essential for proper immune cell function. ADCC can destroy virally infected cells and cancer cells, and is thus a target for monoclonal antibody (mAb) therapies (53). ADCP is also an important mechanism in mAb therapy targeting malignant cells (14). ADCC and ADCP are dependent on the ability of low to moderate affinity FcγRs to cluster on fluid plasma membranes for activation to occur (54) (**Figure 1**). Equally important is the regulation of these receptors when no activation signal is present.

Proper activation of FcγRs following Fc engagement by macrophages requires clustering of FcγRs and the displacement of inhibitory receptors. In one study utilizing murine RAW 264.7 cells, segregation of CD45, a phosphatase responsible for dephosphorylating ITAMs, is dependent on antigen distance from the target membrane (55) (**Figure 1**). It appears that if the antibody is >10 nm from the target surface, there is a substantially impaired ADCP response. This phenomenon is due to the location of the epitope; epitopes closer to the surface exclude the inhibitory CD45 molecule (which stands ∼22 nm tall vs. FcγR-IgG complex = 11.5 nm) from the immune synapse. Interestingly, a follow-up study that focused on FDA-approved mAbs found the targets were small surface proteins (<10 nm in height) suggesting there may be a requirement for mAb epitopes to be located close to the surface for therapeutic efficacy. CD45 was also excluded from the immune synapse in activated human T cells (56). Another study concerning inhibitory module segregation on human macrophages demonstrates CD64, but not CD32a, and inhibitory signal regulating protein α (SIRPα), in conjunction with CD47 (a receptor that inhibits macrophage phagocytosis), are clustered on quiescent cells but upon activation segregate in a process regulated by spleen tyrosine kinase (SYK)-dependent actin cytoskeleton reorganization (57). Recently, FcγR diffusion has been shown to be inhibited by the CD44 transmembrane protein which is immobilized by linearized actin filaments via ezrin/radixen/moesin (ERM) and binds hyaluronan in the glycocalyx (58). This study used primary human macrophages as well as murine cell lines and murine models, utilizing single particle tracking found CD44 and hyaluronan decreased the diffusion rate of FcγRs, while also sterically blocking the binding of FcγRs to immune complexes (**Figure 1**).

Receptor clustering overwhelms constitutive inhibition as described previously, allowing phosphorylation of the ITAM. ITAMs are phosphorylated via SYK, Src family kinase (SFKs) or ζ-chain-associated protein kinase 70 (ZAP-70) for downstream activation of phosphoinositide-3-kinase (PI3K), NF-κB, extracellular signal regulated kinase (ERK), phosphatidyl inositol 4-phosphate 5-kinase γ (PIP5Kγ), GTPases and other SRC-family kinases (53, 54, 59, 60). Along with FcγR clustering, actin polymerization and depolymerization is equally important for phagocytosis in RAW 264.7 macrophages by creating lammellipodium/pseudopods. These protrusions are controlled by Rac GTPase and lipid composition (54, 59) (**Figure 1**). Clustering has also been observed on the plasma membrane

of murine derived macrophages using total internal reflection microscopy (TIRF) of a lipid bilayer supporting IgG (61). The FcγR microcluster appears on the macrophage pseudopod edge and is subsequently transported to a synapse-like structure thereby recruiting SYK and production of PtdIns(3–5)P3 coordinated with lamellar actin polymerization. Another study on quiescent human macrophages found lateral diffusion of FcγRs is regulated by tonic activity of SYK causing actin cytoskeleton organization to increase the likelihood of FcγRs to be pre-clustered upon finding a pathogen (62).This study further described differential FcγR mobility upon activation. FcγRs at the periphery of the actin-rich pseudopod were more mobile than those already immobilized by binding of IgG-rich regions. The authors explained that this mobility difference is controlled by SYK-mediated regulation of the actin-cytoskeleton which would increase the likelihood of FcγRs to engage more IgG molecules at the leading edge of the lamellipodium/pseudopod and not waste time diffusing into already IgG-dependent, FcγR-immobilized, actin-rich rich regions of plasma membrane. Mobility of FcγRs was described earlier to be decreased at the trailing end of polarized macrophages by CD44 that was bound to linear actin and connected to hyaluronan (58). It was also found in this study that on the leading edge of polarized macrophages, the side that encounters opsonized material, Arp2/3-driven actin branching predominates, initiated by phosphotidlyinosotide (3–5)-trisphosphate production, and increased FcγR mobility allowing for more efficient clustering at the immune synapse. When Arp2/3-driven actin branching predominates, it was found CD44 is more mobile allowing greater FcγR mobility (**Figure 1**).

In the human NK92 cell line, transduced to express CD16a, a study showed β2 integrins mediate the dynamics of FcγR receptor microclusters in a protein-tyrosine kinase 2 (Pyk2)-dependent manner, controlling the rate of target cell destruction by ADCC (63). β2 integrins bind ICAM-1 on the target cell allowing adhesion and signal transduction through Pyk2 for actin remodeling and the subsequent enhancement of FcγR mobility. Furthermore, sites of granule release are surrounded by clusters of CD16a and release points are devoid of actin. Human NK cell lytic granules also converge at the surface in a dynein and integrin-signal dependent manner which aids spatial targeting of the weaponized molecules to limit off-target damage (64). Surprisingly, CD16a is essential for ADCC of human CD16+ monocytes and upon CD16a engagement, β2 integrins are activated along with TNFα secretion thereby indicating that non-classical monocytes (CD16+) are the sole monocyte class capable of ADCC (65).

During the early stages of phagocytosis by RAW 264.7 cells, direct contact between FcγRs and IgG is increased by greater IgG density on particles, and increased IgG density results in an increased level of early signals. However, late stage signals are "all or nothing," not concentration dependent, and regulated by PI3K concentration in the phagocytic membranes (66). In this study, low IgG density decreased the amount of opsonized particles but not the rate of phagosome formation and low IgG density particles that did result in phagocytosis recruited the same amounts of late stage signaling molecules (PIP3, Protein kinase C ε type, p85 subunit) and actin. Overall it appears that FcγRs control the initial binding process essential for scanning the foreign particle and initial activation by binding IgG and later stages of commitment to destruction of the particle are controlled by both IgG density and membrane lipid composition.

On murine and human macrophages, receptor clustering upon activation is consistent with a change in the heterogeneity of the membrane lipid composition to a highly ordered phagosomal membrane that is heavily enriched in sphingolipids and ceramide but lacking cholesterol (67) (**Figure 1**). The authors state that lipid remodeling mediates F-actin remodeling and the biophysical characteristics of the phagosomal membrane are essential for phagocytosis. On human B cells, a polymorphism of the inhibitory receptor CD32b (Ile232Thr) located in the middle of the transmembrane domain, is described to decrease inhibitory function (68). This mutation was shown to result in aberrant localization to a sphingolipid and cholesterol rich region in contrast to the Ile232 wild-type. Aberrant localization is not surprising considering the introduction of a polar residue into the transmembrane domain (69). Furthermore, the ability of CD32b to inhibit B cell receptor (BCR)-mediated PIP3 production, AKT, phospholipase C-γ-2 (PLCγ2) activation and calcium mobilization was impaired in cells expressing the CD32b Thr232 allotype as compared to Ile232. The authors indicate the FcγR locus was associated with SLE and this polymorphism may promote disease. Thus, it appears lipid composition is important for FcγR-mediated mechanisms.

The unique construction of CD16b indicates the potential for a different activation mechanism for neutrophils. Neutrophils predominantly express CD16b with 10-fold less CD32a. CD32a signal transduction is well described and thought to be the canonical FcγR signal transduction via phosphorylation of ITAMs and subsequent SYK recruitment (70). However, CD16b contains a GPI anchor and does not have a polypeptide transmembrane domain nor is it known to associate with a signaling coreceptor, therefore, it is unclear how CD16b promotes signaling in neutrophils (**Figure 2**). CD16b plays a role in the initial binding of immune complexes in concert with β2 integrins (71). Currently there are conflicting studies suggesting that CD16b can transduce a signal on its own (70, 72, 73), or it transduces a signal by acting with CD32a (74). A recent study found CD16b cross-linking increased IL-10 and TNFα expression, phosphorylated SHP-2 in a lipid-raft mediated manner and inhibited apoptosis in neutrophils. Lipid composition certainly may be an important part of CD16b signal transduction in mechanisms similar to those discussed previously for macrophage phagocytosis and CD32b on B-cells, however the role of lipids in neutrophil activation is not understood (75–81). Interestingly proteinase 3 (PR3), CD16b, cytochrome b558, and NADPH oxidase co-immunoprecipitate on lipid rafts and PR3 and CD16b colocalize in confocal imaging suggesting these may interact in a lipid raft (75). Other findings suggest CD16b signals in conjunction with CR3 via lectin-like interactions (82), leading to neutrophil respiratory bursts (72). The function of GPI-linked CD16b remains undefined despite the high abundance of CD16b in the body and critical roles in mAb therapies (83).

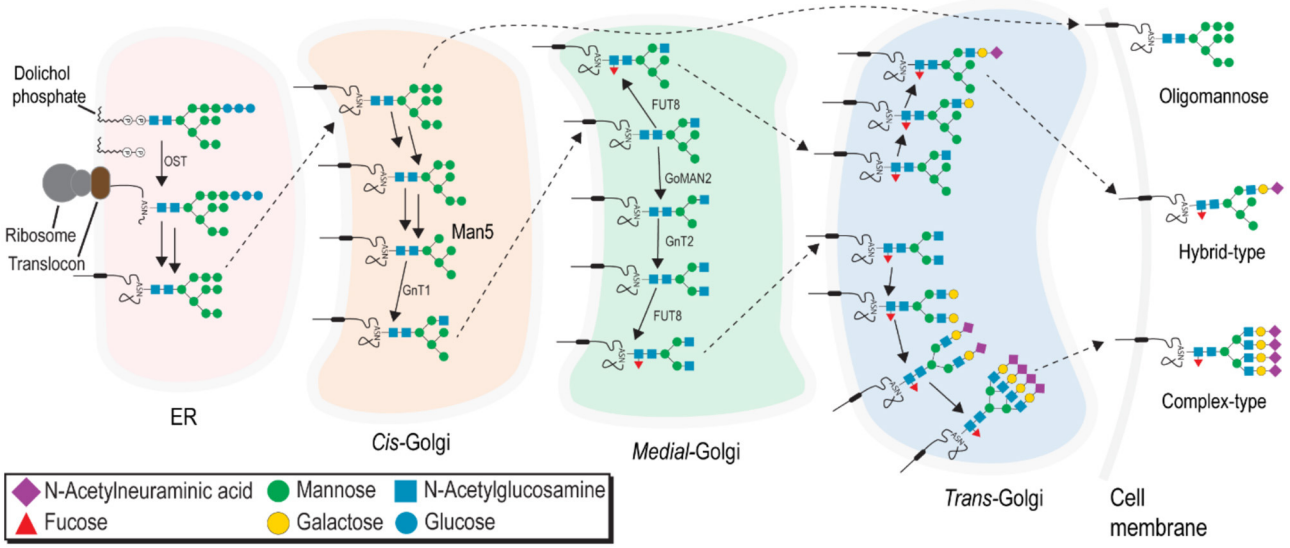


FIGURE 3 | N-glycans are processed in the ER and Golgi to produce three major classes: minimally processed oligomannose-type N-glycans, fully processed complex-type N-glycans, and hybrid-type N-glycans with an intermediate level of processing.

The Type of FcγR Membrane Anchor Impacts Activation

There are clear differences between the signaling and antibody-binding affinity of soluble and membrane-anchored FcγR forms. However, less is known about the effects of the specific FcγR membrane anchors on affinity and cell activation. All FcγRs are localized to the membrane by a transmembrane polypeptide moiety or a glycosylphosphatidylinositol (GPI) moiety (CD16b only) (**Figure 2**). A micropipette adhesion assay demonstrated CD16a attached to microspheres via a GPI anchor bound roughly 5-fold tighter to IgG1-coated red blood cells (RBCs) than CD16a tethered by a transmembrane domain (84, 85). Interestingly, it also appears IgG1-coated spheres treated with phosphoinositide phospholipase C (PIPLC) to remove the diacylglycerol moiety bound to GPI-linked CD16a with 12-fold less affinity. These authors observed a 60-fold decrease when the GPI-anchor was completely removed. A CD16b-GPI construct showed 2-fold decrease of affinity upon PIPLC treatment and an 11-fold decrease following removal of the GPI-anchor. The authors hypothesized that enhancement of binding affinity associated with the GPI anchor may be due to an allosteric effect on CD16, changing the structure to bind IgG more effectively; such an allosteric mechanism was observed with other GPI-anchored proteins (80). Further studies will be required to fully elucidate how the GPI-anchor affects CD16b and how specific aspects of the membrane anchor confers distinct properties *in vivo*.

POST-TRANSLATIONAL MODIFICATION OF THE ANTIBODY AND RECEPTOR

Asparagine-linked (N-) glycosylation is one of the most common protein modifications performed by the eukaryotic cell and is a substantial modification of all FcγRs [**Figure 2**; for a thorough review of N-glycan processing, see (86)].It is important to note, however, the resulting glycans processed in the ER and Golgi can be grouped into three distinct forms: (1) minimally-processed oligomannose type N-glycans, (2) intermediate processed hybrid-type N-glycans with processing on one of the two core mannose branches, and (3) highly-processed complex-type N-glycans with extensively modified branches (**Figure 3**).

Several variables introduce a significant degree of heterogeneity into the N-glycan present at any single site on a glycoprotein, ranging from substrate availability, protein anchor type, to accessibility of N-glycan site, potentially creating a vast diversity of protein forms and functions (87–90). This heterogeneity also renders glycoproteins challenging targets for *in vitro* studies to characterize structure. Minimally-processed hybrid and oligomannose type N-glycans are not expected at the cell surface because these forms harbor terminal mannose residues that may bind to the mannose receptor and elicit an immune response (91, 92). Though many previous glycomics studies report high levels of oligomannose N-glycans recovered from primary cells, the abundance of these under-processed forms is likely due to cell lysis and recovery of unprocessed glycans from the ER. If under-processed forms are present on the cell surface, these must be protected from binding to the mannose receptor. Therefore, highly processed complex-type N-glycans are expected as the predominant species at the cell surface.

The functional impact of N-glycosylation at the conserved asparagine 297 residues in IgG1 is well established. IgG1 glycosylation at Asn-297 is essential for the IgG-FcγR interaction (93). The N-glycosylation profile of serum IgG changes due to multiple factors, including age, gender, infection, pregnancy, and disease (94–97). The variation in IgG1 Fc glycoforms is known to change antibody affinity toward the

FcγRs (98), and this fact has also been leveraged to develop glycoengineered mAbs and anti-inflammatory glycoforms of intravenous immunoglobulin (IVIG) (99, 100). The wealth of knowledge regarding IgG glycoforms is due in large part to protein abundance and ease of obtaining samples. However, little is known about the glycosylation of FcγRs on immune cells.

FcγRs are heavily glycosylated molecules, containing two to seven N-glycans (**Figure 2**). The extent of FcγR modification was evident as early as 1988 as certain FcγRs from native tissue migrated much slower in SDS-PAGE gels than expected based on the polypeptide mass alone. Furthermore, the migration rate increased after treatment to specifically remove N-glycans (101, 102). There is a prominent gap in knowledge about the impact of FcγR N-glycosylation on immune function largely due to limited studies of the native FcγRs purified from primary leukocytes. However, it is known that CD16a expressed by NK cells had a distinct N-glycosylation profile when compared CD16a expressed by cultured monocytes, though this determination was made using lectin binding (103) and surface CD16a on the NK cell and monocyte displayed differential antibody-binding affinity that was attributed to differences in cell-specific CD16a N-glycosylation (104).

Even though native glycoforms of all FcγRs are not known, the effect of N-glycosylation on binding affinity has been well characterized *in vitro* using protein expressed with mammalian cells. Aglycosylated, recombinant, soluble (s)FcγRs bind IgG1 Fc at different affinities than glycosylated forms, thus the IgG-FcγR interaction is sensitive to receptor N-glycosylation (105–107). Recent studies reported substantial differences in affinity for sFcγRs expressed in recombinant systems (106, 108–110), N-glycosylation profiles of NK cell CD16a and soluble CD16b from serum revealed surprising heterogeneity and substantial differences from recombinantly-expressed protein (109, 111).

Specific CD16a Glycoforms Bind Antibody With High Affinity Comparable to CD64

The analysis of N-glycan composition from FcγRs provides a characteristic profile of a protein (112). Glycomics analysis of CD16a on circulating NK cells from three healthy donors revealed a surprising abundance of under-processed forms (∼45% hybrid and oligomannose-type N-glycans). CD16a is N-glycosylated at five sites (**Figure 2**). The remainder of the N-glycans were primarily complex type, biantennary N-glycan structures with a high degree of sialylation (78%) and fucosylation (89%) (109). The under-processed forms do not likely originate from unprocessed CD16a in the ER because all of the observed hybrid forms were sialylated, a modification that occurs in the late Golgi compartments (113) (**Figure 3**). Moreover, the presence of oligomannose type N-glycans on CD16a from almost all recombinant sources suggests that restricted processing is a conserved feature (108–110). N-glycans at Asn38 and Asn74 were not observed using this glycomics approach to study NK cell CD16a; perhaps these large glycans ionize too poorly to be observed in a derivatized form, but robust ionization of the peptide provides measurable signals for CD16b N38 and N74 glycopeptides (111).

Recombinant expression has thus far failed to generate CD16 with glycan profiles matching those measured for CD16a or CD16b from primary cells. CD16a is the most heavily studied FcγR due to its role in ADCC and the associated therapeutic applications. Glycomics characterization of soluble extracellular domain of CD16a (sCD16a) from HEK293, NS0, and CHO cell lines showed stark differences when compared to CD16a from NK cells, including a high abundance (over 90% compared to 55% in NK cells) of biantennary and triantennary complex type N-glycans with low levels of sialylation (108–110). Moreover, each recombinant system has the potential to synthesize unique N-glycan structures that are not commonly found on native human proteins, such as LacDiNAc (GlcNAc-GalNAc) from HEK293 cells, α-Gal epitopes (αGal-βGal-βGlcNAc), terminal N-acetylglycolylneuraminic acid in NS0 cells and only α-2,3 linked sialic acids in CHO cells (106, 114). These terminal modifications can potentially alter the binding affinity to IgG in an unexpected and undesirable manner.

Differences between native and recombinant CD16a processing render studies of binding affinity using recombinant material suboptimal, however, these materials still represent the best option for many *in vitro* studies. Furthermore, binding affinity measurements have utilized the soluble extracellular FcγR domains due to challenges associated with extracting full-length material from the membrane. Tethering CD16a to the membrane changes the N-glycosylation, likely due to differential localization within the Golgi (88, 109). Unfortunately, the N-glycosylation profile of full-length CD16a (frCD16a) expressed with HEK293 cells revealed an N-glycan profile unlike that found on NK cells (109). N-glycans from frCD16a showed less under-processed oligomannose and hybrid types (27% in frCD16a and 45% in NK cell CD16a) and the complex-type N-glycans were highly branched. Thus, cell-type specific glycosylation accounts for the dissimilar N-glycan profile on CD16a from primary and recombinant sources and impacts binding affinity measurement, as discussed below.

Recombinant FcγRs are valuable to characterize the role of N-glycosylation on IgG binding affinity, despite clear differences in N-glycan processing when compared to endogenous material. One recent study reported a 40-fold increase in affinity toward afucosylated IgG1-Fc (G0 form) when complex type N-glycans on CD16a were replaced with Man_5 N-glycans (110). This gain revealed that CD16a can bind with an affinity comparable to CD64, the "high affinity FcγR." A comparable study demonstrated that higher amounts of larger sialylated complex type N-glycans on CD16a expressed in CHO cells correlated with lower affinity for Rituximab (108).

Of five CD16a N-glycans, only two appear essential for high affinity interactions. Mutating the protein to eliminate N-glycan addition with N45Q and N162Q substitutions reduced the affinity for IgG1-Fc (109, 115–117). However, the reported influence of N-glycan composition was primarily driven by the N-glycan at N162: only the N162Q mutation abolished the affinity gain due to Man_5 N-glycans on CD16a (110). These observations are in agreement with the fact that glycans at N45

and N162 form interactions with the CD16a polypeptide and influence protein structure (118) and glycans at these two sites showed the greatest restriction in N-glycan processing using the HEK293 and CHO systems (119). Thus, cell-type specific CD16a N-glycosylation patterns influence affinity for IgG1 and a range of potential affinities are accessible purely through modifying N-glycan processing.

N-glycosylation of CD16b

CD16b is a highly similar paralogue of CD16a and only found in humans (97% sequence homology of the extracellular antibody-binding domain). However, two common CD16b alleles encode either four (NA1) or six (NA2) N-glycosylation sites (120, 121) (**Figure 2**). Considerable site-specific diversity in N-glycan structures was present on sCD16b obtained from 2l of pooled human serum (111). Serum sCD16b is generated by ADAM17 cleavage of cell surface CD16b upon neutrophil activation (48). Thus, sCD16b were likely membrane bound when the N-glycans were being processed. The N-glycans at each site had unique profiles ranging from smaller oligomannose type N-glycans at N45 to large complex type N-glycans with extensive elongation, sialylation, and fucosylation at N38 and N74, unlike sCD16b expressed in recombinant systems (106, 114, 122). Additionally, allele specific (NA1 and NA2) N-glycosylation profile at N162 and N45 of donor matched serum and neutrophil CD16b confirmed the observations of CD16b from pooled serum, revealing moderate variability in the abundance of the most prominent glycoforms (123). The profile of sCD16b from serum was distinct from CD16a expressed by NK cells that displayed a greater level of under-processed N-glycans (109, 111). The presence of oligomannose type N-glycans only at N45 strongly suggests under-processing of N-glycan is restricted to a single site on the protein with as many as six N-glycosylation sites (111).

The stark differences in the glycosylation profile of sCD16b from serum compared to recombinant sCD16b further emphasized the importance of cell type specific N-glycosylation. Glycomics analysis of CD16b from HEK293, NS0 and BHK revealed mainly multiantennary complex type N-glycans with a high degree of sialylation and fucosylation (106, 114, 122). The N-glycosylation profile of recombinant sCD16a and sCD16b are comparable as most of the N-glycosylation sites are shared (124). There was a minimal difference (2-fold increase) in affinity when sCD16b-Man5 binding to IgG1-Fc (G0F form) was compared to sCD16b with complex-type N-glycans (110). This was surprising considering that the extracellular antibody binding domains of CD16a and CD16b (NA2) differ at only four amino acid residues. Moreover, both CD16s are functionally distinct because CD16a-complex type has a 15-fold greater affinity for IgG-Fc than CD16b-complex type (110). The affinity and sensitivity to glycan composition for CD16b was improved to that of CD16a by mutating a single residue, Asp129, to Gly based on the CD16a sequence (124). The authors demonstrate with x-ray crystallography and molecular dynamics simulations that Asp129 buckles the CD16b backbone upon binding IgG1 Fc. Thus, buckling shifts a nearby residue, Arg155, which makes a different contact with the N162-glycan that is not observed in CD16a.

N-glycosylation of CD32

The N-glycosylation profiles of sCD32a and sCD32b expressed with recombinant systems were highly comparable (106, 108, 114). There are two to three N-glycosylation sites on CD32: CD32a (3), CD32b (2), and CD32c (2; 32b and 32c have identical extracellular domains) (**Figure 2**). Glycomics analysis of CD32a and CD32b expressed in HEK293, NS0, and CHO displayed predominantly biantennary and triantennary complex type N-glycan structures with a low degree of sialylation and varying levels of fucose (106, 108, 110, 114). Binding affinity between sCD32a and sCD32b was comparable and neither appeared sensitive to N-glycan composition as sCD32(a or b)-Man5 and sCD32(a or b)-complex type bound IgG1 Fc with similar affinities (106, 110). CD32a polymorphisms (R131 or H131) cause differences in binding to IgG subtypes, potentially changing the sensitivity of immune complexes to phagocytosis by neutrophils and monocytes (121, 125). However, N-glycan analysis on the receptor expressed in CHO cells showed no substantial difference in glycosylation pattern between the two CD32a allotypes (108). The site-specific N-glycosylation profile and native N-glycosylation profile for any CD32 is not currently available.

N-glycosylation of CD64 Also Impacts Binding Affinity

The high affinity FcγR, CD64, is distinct from other FcγRs because it contains an additional extracellular domain (126). Moreover, CD64 can potentially receive N-glycosylation modification at seven sites in its extracellular domain (**Figure 2**). A comparative glycomics analysis of the sCD64 expressed in HEK293, NS0, and CHO cell lines showed biantennary and multi-antennary complex type N-glycans with varying degrees of sialylation and fucosylation as the most abundant glycoforms (106, 108, 114). A distinct feature which was conserved across sCD64 expressed in all three cell lines was the higher abundance of oligomannose structures when compared to recombinant CD16 or CD32. It was speculated that the presence of Man_5 forms (the most abundant oligomannose N-glycan in these cell types) conferred a stabilizing effect toward IgG1 binding since the higher abundance of Man_5 forms (14.4% in NS0 and 5.2% in CHO) correlated with an increase in binding affinity to Rituximab (108). According to the authors, the increased affinity was due to the lack of core fucose on the Man_5 structure which can potentially prevent steric hindrance effects similar to that observed in fucosylated N-glycan on IgG1 (115, 127). The authors also observed that the presence of large sialylated complex type N-glycans on CD64 correlated with reduced binding affinity for Rituximab, indicating that these glycans destabilized the interaction (108). Even though N-glycan composition on CD64 can affect IgG1 affinity, the N-glycosylation profile of native CD64 and the composition of N-glycans at each site remains unknown.

N-glycosylation processing depends on the amino acid sequence and secondary structures which affect the exposure of substrate monosaccharide residues to the glycan processing enzymes (**Figure 3**). Presence of both the under-processed and

highly-processed (tetraantennary sialylated) N-glycan structures on NK cell CD16a and recombinant sCD64 suggests site-specific glycan modification. Oligomannose structures at specific sites on sCD16a have been implicated in modulating IgG affinity; similarly, specific sites on CD64 can be involved in modulating CD64-IgG1 affinity (108, 110). Thus, a thorough analysis of site-specific N-glycosylation analysis of recombinant and endogenous FcγRs from all expressing tissues is required to fully elucidate the role of N-glycosylation pattern at specific sites in affinity modulation.

HOW MULTIVALENCY IMPACTS IgG-FcγR INTERACTIONS

Investigating factors that contribute to the monovalent affinity of IgG-FcγRs interaction revealed clear differences in the affinity of antibody subclasses for certain receptors, however, multivalent avidity likely determines the *in vivo* immunological response initiated by these interactions. High IgG concentrations in the serum of ∼10 mg/ml provide monomeric antibody to the receptors at a concentration of ∼67 μM, vastly exceeding the K_D of IgG1 for all human receptors (7). Thus, surface-borne FcγRs are occupied on cells circulating in the peripheral compartment and multivalent interactions must compete with monomeric IgG to cluster receptors (7, 8). Receptor cross-linking and clustering on the effector cell surface is essential for signal transduction through FcγRs, thus multivalent immune complexes or opsonized targets are the functionally appropriate ligands for the receptors (**Figure 1**) (54, 128). Distinct FcγRs are engaged depending on the responding cell type, the IgG subclass, the antibody concentration on the opsonized target, and the size of immune complex (**Figures 1**, **2**) (129, 130). Furthermore, the differential binding of immune complexes has therapeutic as well as pathogenic properties, especially during infection and autoimmune disease but not all aspects are well-defined (9, 15). Therefore, defining the critical factors associated with immune complex recognition is required to fully understand the antibody-mediated immune response.

Immune Complex Size Determines Effector Function

The importance of interactions between multiple monovalent ligands and multiple receptors is well known, however, the study of multivalent interactions remains challenging. Early attempts to generate multivalent immune complexes through heat aggregation of IgG produced aggregates with varied valency, immunogenicity and ill-defined sizes (131, 132). Technological advances in recent years produced immune complexes of defined size and valency which accurately represent those generated *in vivo* (130). Functional interrogation using defined immune complex revealed that immune complex size contributes to interactions with FcγRs.

Immune Complex Size Affects Binding

The concentration of antigen-specific antibody in the serum and likewise immune complex size is expected to change during an immune response, and size-associated changes in the immune response are well described (130, 133). Nimmerjahn and coworkers used well-defined immune complexes formed by all four IgG subclasses binding to FcγRs expressed on a CHO cell surface to systematically determine that there was a clear size-dependent gain in binding by IgG2 and IgG4 immune complexes and the size of an immune complex can overcome IgG glycan truncation, a modification that destroys the monovalent interaction (134). Moreover, the binding patterns were comparable to experiments using primary leukocytes that increased cytokine secretion in response to larger immune complexes. These data led to a mathematical model that describes effects of valency and IgG subclass on *in vivo* function (135). The differential binding due to a change in the size of immune complex can potentially lead to substantial changes in cell signaling and recent technical advances provide a means to quantitate signaling with cell-based assays (136).

Role of Immune Complex Size in Autoimmune Disorders

The formation of immune complexes with soluble self-antigen is implicated in the pathophysiology of several autoimmune diseases (137). IVIG is a frequent treatment for a variety of autoimmune disorders, but the exact mechanism of action is not known (138). Even though there is a well-documented role of CD32b in decreasing an immune response triggered by autoantibody immune complexes in murine model of immune thrombocytopenia (ITP) (139), a recent study demonstrated that engaging the inhibitory CD32b alone is not responsible for the decrease in phagocytosis of RBC opsonized by autoantibody in human ITP patients. Instead, the direct engagement of IgG by CD64 and CD32a caused the decrease in phagocytosis (140). Surprisingly, though IVIG dimers and multimers are not necessary for therapeutic efficacy in murine models for ITP, small IVIG oligomers provided more potent inhibition of phagocytosis, indicating a role of IVIG immune complexes in blocking pathogenic immune complexes from binding to activating FcγRs (141). Consistent with this observation, immune complexes formed with the anti-citrullinated protein antibodies isolated from rheumatoid arthritis patients bound preferentially to activating and not inhibiting FcγRs expressed on CHO cells (142). Moreover, CD64 on activated neutrophils and CD32a on macrophages were recognized as receptors for the autoantibody immune complex, eliciting the secretion of pro-inflammatory cytokines. These observations formed the basis for developing engineered multivalent immune complexes as therapeutic options.

Considerations Regarding Immune Complex Size in Therapeutic Development

Multivalent synthetic immune complexes show promise and may prove useful in the clinic. For example, a trivalent IgG-Fc construct inhibited autoantibody-mediated FcγR-dependent cellular responses in primary human cells and autoimmune murine models (143). Likewise, an engineered hexameric-Fc construct bound to primary differentiated human macrophages and triggered internalization, colocalizing

with the activating FcγRs and elicited a decrease in the phagocytosis of antiCD20-coated human B cells and platelets in a murine ITP model (144). The hexameric Fc construct did not trigger internalization of CD32b and exhibited a much shorter serum half-life in animal models than IgG1, however, the inhibition was effective for several days after the initial injection, suggesting a potential for clinical use. In contrast to the approach of preventing the internalization of pathogenic immune complex to block phagocytosis of healthy cells or activating a pro-inflammatory response, a designed bispecific antibody formed larger complexes that neutralized soluble antigens, leading to rapid clearance from serum of a murine model (145). Thus, studies of multivalent IgG-FcγR interactions provide guidance for the development of effective therapeutic options. However, there are multiple antibody and antigen associated factors which govern the antigenicity of immune complexes that must be considered when designing antibodies with defined FcγR-dependent functions.

Features of the Antibody and Antigen That Impact Antigenicity of the Immune Complex *in vivo*

The Ratio of Antibody to Antigen

Antibody concentration relative to antigen changes throughout the progression of an immune response against an infectious pathogen. Considering influenza infection as an example, the B-cell response can take up to 7–14 days to produce antibodies (146). Generally, the antigen-specific antibody titers increased by up to 10.2-fold, depending on the patient, vastly changing the antibody to antigen ratio and the antibody production can be sustained or subside depending on clearance of the organism.

A minimal threshold of antibody density must be surpassed to elicit an immune response during encounters between an opsonized target and effector cell, typically seen during pathogenic infection (147, 148). Antibody concentrations that exceed the threshold lead to an increase in phagocytic activity, as demonstrated by primary mouse bone marrow derived macrophages phagocytosing opsonized sheep erythrocytes. Moreover, at relatively high concentrations of IgG, a valency dependent induction of IL-10 production was seen (148). Similarly, infection with *Cryptococcus neoformans* in mice could be cleared using a specific ratio of antibody to antigen, ratios with excessive antibody led to a detrimental host response mainly due to a reduction in pro-inflammatory cytokines secretion in organs associated with the infection (149). Apart from changes in cytokine secretion potential, larger immune complexes formed with high concentrations of neutralizing antibody against dengue virus actually inhibited antibody-dependent enhancement by binding to the inhibitory receptor CD32b on phagocytic monocytes (150). Thus, relative antibody concentration can modulate immune response in an FcγR-dependent manner by altering the size and concentration of immune complexes; this effect may be similar to the therapeutic benefit of IVIG in autoimmune conditions.

Concentration of the Immune Complex

Immune complex concentration likewise impacts viral infection. Apart from the traditional view of Fab-mediated neutralizing activity, Fc dependent effector functions are becoming increasingly recognized in protection against viral infection (16, 17, 151). Classical FcγR-dependent protective mechanisms such as ADCC and ADCP, as well as antibody dependent enhancement of infection, are influenced by the size of the immune complex and IgG subtype coating the viral particle (17, 152). The production of a high concentration of immune complexes are common during chronic viral infection in mice (153). However, high concentrations do not always lead to favorable outcomes. A high concentration of immune complex blocked FcγRs on primary murine macrophages and dendritic cells, negatively impacting viral clearance, and other FcγR-related activity (153). These phenomena were independent of CD32b and reversed once the immune complex concentration was reduced. Thus, the role of FcγRs during pathogen infection is complex and varied but there is a clear dependence of cellular response based on immune complex size and concentration, similar to that observed in autoimmune disease discussed above.

Affinity of the Antibody for Antigen

At a fixed antibody concentration, the affinity of the antibody toward the antigen can determine how many Fcs are displayed on the immune complex and are available to interact with FcγRs (154). A recent study showed that at saturating concentrations, antibodies with high affinity for antigen elicited a weaker ADCC response compared to antibodies with lower affinity (K_D = 0.8 nM and 72 nM, respectively) (155). The observed difference in the immune response was attributed to the higher proportion of monovalent antigen binding displayed by the lower affinity antibody, recruiting a larger number of antibodies to the cell surface and increasing the number of Fcs available to the leukocyte. A notable feature of this observation is the initial IgG response often produces antibodies with antigen-binding affinities similar to the lower affinity antibody in this study. Antibody concentration and antibody-antigen affinity are not the only factors affecting immunogenicity of immune complex. A comparative analysis of three anti-TNFα antibodies with a range of affinities (K_D = 0.18–5.1 nM) showed that the size and composition of the immune complex was determined by the properties associated with epitope location and binding energetics (156).

Epitope and Antigen Location

Location of the epitope influences the immune response. Neutralizing antibodies targeting the stalk region of the influenza hemagglutinin protein induced FcγR-dependent cytotoxicity while antibodies binding the head domain did not (12). A comparable analysis of anti-Ebola antibodies showed that binding to the most membrane distal portion of viral surface glycoprotein elicited the highest ADCP and antibody-dependent neutrophil phagocytosis (ADNP) compared to antibodies that bound to the membrane proximal regions (157). Even though epitope location on the antigen is not directly implicated in changes in immune complex size in these studies, it is likely

that the epitope location causes changes in immune complex properties since three different monoclonal antibodies against different epitopes on sCD154 and TNFα also formed different immune complexes (156, 158). In other cases, the height of the antigen from the target surface affected phagocytosis in a valency-independent manner (55). Antigens which are <10 nm from target surface promoted phagocytosis when compared to antigens further away from the surface because close contact between target and effector cell surface was necessary to exclude effector cell the inhibitory CD45 from the immune synapse following FcγRs clustering (as noted above). Additionally, antibodies binding West Nile virus epitopes that are normally buried can form immune complexes, given sufficient incubation time, though these immune complexes are smaller and led to lower neutralization levels (154). Thus, location of the epitope can affect the immune response but the effect of epitope location on immune complex size is not fully understood.

The location of the antigen (soluble or cell bound) affects FcγR clustering and the subsequent immune response. A soluble antigen may form relatively smaller immune complexes which are endocytosed but a cell surface antigen forms a relatively larger opsonized target that is more likely phagocytized as determined using mouse bone marrow-derived macrophages (133). Both mechanisms, triggered through FcγRs, are distinct and induce different signaling and subsequent immune responses (128, 159). In one example, small soluble immune made with soluble CD154 would be expected to be endocytosed, and CD154 tethered to a T cell membrane led to the formation of very large complexes at the cell surface (158). Surprisingly, the specific monoclonal antibody greatly influenced the immune complex structure. It is also known that opsonized targets can exhibit lateral diffusion on the leukocyte surface which also affects the multivalent interaction with FcγRs (160).

Malleable vs. Rigid Target Surfaces

In addition to size and shape, deformability of the target also impacts activation. The phagocytosis of opsonized polyacrylamide beads tuned to exhibit different rigidity established that phagocytosis of ridged particles was preferred over relatively more deformable particles by mouse bone marrow-derived macrophages (161). A related study demonstrated that murine macrophage RAW264.7 cells phagocytosed emulsion droplets at a lower IgG concentration when compared to solid particles (162). It was speculated that the attachment of IgG on the surface of rigid particles prevents the lateral diffusion of opsonizing antibodies, while lateral diffusion was observed in opsonized emulsion droplet. Thus, the location of the antigen, which facilitated higher cell surface FcγRs interaction at lower antibody concentrations, can affect recognition of the complex.

IgG-Subclass Impact Immune Response

FcγR binding is also affected by IgG subclass. Specificity of a specific IgG subclass binding to a FcγR is largely studied in context of a monovalent interaction (23), however, immune complexes and opsonized target cells are the natural ligands. Additionally, specific IgG subclasses are related to various disorders indicating immune complex composition is important (1, 152, 163). Therefore, studying these interactions in a multivalent form is required to accurately determine their binding properties and the subsequent immune response. The observation that immune complexes of certain IgG subclasses only bind at higher concentrations indicates that IgG subclass is also a variable which can affect the immune response (164).

The Fcs of different IgG subclasses have distinct amino acid residues and hinge regions which can affect binding to the FcγRs, despite a high degree of sequence conservation (**Figure 2**) (1). A systematic analysis of multivalent binding for the four human IgG subclasses to the cell surface FcγRs revealed the IgG2 and IgG4 subclasses, which showed minimal affinity in a monovalent interaction, bound as immune complexes to FcγRs expressed on CHO cells at higher concentrations (164). This study also demonstrated that allotype variants of FcγRs had different binding properties toward immune complexes generated by different IgG subtypes. CD16a V158 bound IgG3 immune complexes with high affinity while CD16a F158 bound more weakly and CD32a H131 had a higher affinity to IgG2 immune complex compared to CD32a R131. Another report showed that the CD32a H131 variant bound to IgG1, IgG2, and IgG3 with higher affinity than CD32a R131. This observation may explain why the CD32a R131 allotype is associated with greater susceptibility to bacterial infections and autoimmune disorders (163). Thus, the wide range of binding affinities displayed by FcγRs toward IgG subclass specific immune complexes can impact clinical outcome.

The use of different IgG subclasses in designed immune complexes can also impact potential therapeutic use. Incubation of a hexameric IgG1 Fc construct, discussed above as an inhibitor of phagocytosis, elicited the release of higher cytokine levels in whole blood when compared to PBMCs, likely due to CD16b engagement on neutrophils (not present in PBMCs) (165). Furthermore, the hexameric IgG1 Fc construct also triggered release of cytokines from platelets through a CD32a-dependent interaction. However, a hexameric IgG4 Fc construct did not promote the release of cytokines from neutrophils or platelets. This result is consistent with the reduced affinity of IgG4 for CD16b and CD32a when compared with IgG1, highlighting the potential utility of specific FcγR interactions.

SUMMARY

The multitude of factors influencing the immune system each affects a wide range of responses. This review covers a relatively limited collection of variables that contribute to an FcγR-dependent immune response (**Figure 1**). There appear to be few inviolable laws governing this aspect of the immune system, and every newly discovered variable introduce a new handle to tune the immune response, at least *in vitro*. It is well known that different monoclonal antibodies to a single target elicit different responses, in many cases through the mechanisms described here. If any lessons are to be learned, it is that each antibody must be thoroughly evaluated using systems that recapitulate as closely as possible endogenous immune system components.

One striking example of this tenet is the observation that the efficacy of a hexameric IgG1 Fc increased when neutrophils and platelets were incorporated in an *in-vitro* assay with PBMCs (165). Moreover, soluble complement components can also bind the immune complex to affect the immune response as reported in few studies described above (130, 148, 149). Laboratory studies often focus on immune complexes formed by monoclonal antibodies, but that is likely not the case *in vivo* with a polyclonal immune response to vaccines or infection; one study demonstrated that a mixture of disease neutralizing and disease enhancing antibodies against *Bacillus anthracis* formed immune complexes that elicited a protective immune response (166). Thus, these observations highlight the complex yet important features associated with studying FcγRs function *in vivo*.

Animal models have, and will continue to have, an important role in studies designed to understand human FcγRs in immune function. Despite the differences in FcγR cellular expression patterns and minor differences in binding affinities to human IgG subclass, animal (mainly murine and non-human primate) models have sufficiently recapitulated human FcγR biology to be used for studying FcγR function and test therapeutic molecules (167–173). A recent study determined that the mouse FcγRIV and the human equivalent to human CD16a both share the conserved N-glycosylation site at N162 which mediates tight binding to afucosylated mouse IgG similar to observations in human system, and human IgG binds mouse FcγRs with similar affinity patterns as human FcγRs demonstrating conservation of certain functional features of human FcγR biology in mouse model (170, 174). Furthermore, several studies mentioned in this review have employed murine autoimmune models, humanized models, cell lines or primary cells to test efficacy of engineered antibody products and delineate mechanistic aspects of the FcγRs dependent cellular response, demonstrating that these models are indispensable for understanding human FcγR biology (61, 66, 139, 141, 143, 162). The two successful strategies to attain humanized FcγR mouse models eliminate the influence of mouse FcγRs in studying human FcγR function in these models and can uncover novel role of FcγRs in autoimmune disorders, infection and cancer immunity (175, 176). However, important yet undefined FcγR variables including post-translational modification including glycosylation as well as copy number variation and interaction with coexpressed membrane proteins likely vary in animal models. It is likely that organism diversity in these key variables likewise differentially impacts immune function, comparable to the diversity attributed to protein coding regions and gene variability between species.

It is worth highlighting the role of post translation modification of the FcγRs as another critical variable that is overlooked due to the historical inability to resolve differences in the glycosylation of endogenous material. One future challenge will be matching the level of detail known regarding serum IgG glycosylation with studies of functionally-relevant FcγR modifications as these have the potential to exert an enormous influence on the immune response. Differential gene expression profiles of the glycan modifying enzymes are present in monocytes, dendritic cells, and macrophages, suggesting the potential for the functionally-relevant differentiation and maturation specific N-glycosylation modifications (177). A complete understanding of the immune response will require the definition of these recently discovered variables, with the likelihood that more variables will emerge.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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FcγR-Binding Is an Important Functional Attribute for Immune Checkpoint Antibodies in Cancer Immunotherapy

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T cells play critical roles in anti-tumor immunity. Up-regulation of immune checkpoint molecules (PD-1, PD-L1, CTLA-4, TIM-3, Lag-3, TIGIT, CD73, VISTA, B7-H3) in the tumor microenvironment is an important mechanism that restrains effector T cells from the anti-tumor activity. To date, immune checkpoint antibodies have demonstrated significant clinical benefits for cancer patients treated with mono- or combination immunotherapies. However, many tumors do not respond to the treatment well, and merely blocking the immune suppression pathways by checkpoint-regulatory antibodies may not render optimal tumor growth inhibition. Binding of the antibody Fc-hinge region to Fc gamma receptors (FcγRs) has been shown to exert a profound impact on antibody function and *in vivo* efficacy. Investigation of immune checkpoint antibodies regarding their effector functions and impact on therapeutic efficacy has gained more attention in recent years. In this review, we discuss Fc variants of antibodies against immune checkpoint targets and the potential mechanisms of how FcγR-binding could influence the anti-tumor activity of these antibodies.

Keywords: FcγR, checkpoint blockade, antibody therapy, cancer immunotherapy, IgG isotype

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INTRODUCTION

Immune checkpoints refer to multiple inhibitory pathways that control the immune system to maintain self-tolerance and modulate the intensity of physiological immune responses in order to minimize pathological damage (1–3). Antagonizing antibodies against immune checkpoint inhibitory molecules has achieved great success in cancer treatment (1, 2). However, many tumors do not respond to the treatment, and antibody optimization (especially in the isotype selection) is essential for improving outcomes (4, 5). target-binding specificity, imparted by the antibody's variable region, is well-known to be critical for the primary functional activities of the antibody. However, mounting evidence has shown that the antibody's constant region also plays a crucial role, much of which is mediated through interaction of the crystallizable fragment (Fc) with Fcγ receptors (FcγRs) (6). Fc endows IgG antibodies with effector functions, which include antibody dependent-cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), Induction of cytokines/chemokines and endocytosis of opsonized targets (7).

To date, therapeutic IgG antibodies (either approved or in clinical development) belong to the IgG1, IgG2 or IgG4 subclasses. Each IgG isotype has a distinct binding affinity to the various FcγRs,

which are expressed differently on immune cells. A combination of these features leads to diverse and highly regulated antibody responses.

Antagonizing antibodies against major T-cell inhibitory pathways, such as PD-1/PD-L1 and CTLA-4, have become important parts of cancer therapeutics (1). Consequently, the next wave of therapeutic antibodies targeting alternative immunosuppression pathways (e.g., LAG-3, TIM-3, B7-H3, VISTA, CD73) are rapidly emerging (8). The majority of the immune checkpoint antibodies have low or significantly reduced binding to FcγRs to avoid potential ADCC and CDC, especially when the target molecule is expressed on effector T cells (9). However, for targets such as CTLA-4, TIGIT, and VISTA, competent Fc is required for optimal anti-tumor immune responses in various mouse models (10–12). The mechanisms of action (MOA) may involve the killing of regulatory T cells (Tregs), promoting immune synapse formation and production of pro-inflammatory cytokines due to cross-linking of FcγRs with the competent Fc.

In this article, we summarize the major properties of different IgG isotypes and FcγRs, describe the MOA of different immune checkpoint targets in inhibiting anti-tumor immunity and review the recent studies on the important roles of either binding or not binding to FcγRs in immune checkpoint antibody therapy. It should be noted that many of the findings come from mouse models; the clinical significance of these findings has yet to be determined.

IgG ISOTYPES AND FcγRS

In humans, there are four isotypes of IgG (IgG1-4), differing from the other in their binding profiles to various FcγRs and to complement subunits, such as C1q. IgG1 has the highest affinity to all FcγRs and C1q, leading to significant effector functions, such as ADCC, ADCP, and CDC (5, 13). Although human IgG3 can also mediate competent effector functions, it has a very long hinge region and complex disulfide bonds, resulting in significantly greater polymorphism, which may increase the risk of immunogenicity. Therefore, the IgG3 isotype is rarely chosen in antibody therapeutics (14) and is not further discussed in this review. In comparison, IgG2 and IgG4 induce significantly weaker or no ADCC and CDC (13). The binding features of different IgG isotypes to various FcγRs are summarized in **Table 1** and discussed below.

The overall structures of IgG1, IgG2, and IgG4 are very similar with more than 90% sequence homology. The major differences reside in the hinge region and CH2 domain, which form primary binding sites to FcγRs (19–21). The hinge region also functions as a flexible linker between the Fab and Fc portion.

In addition to differential binding affinity to FcγRs, IgG4, and IgG2 demonstrate other unique features. IgG4 has a unique S_{228} in the hinge region, which allows for interchangeable disulfide bond configurations and formation of "half-antibodies" (22). *In vivo*, IgG4 with different specificity may shuffle, resulting in monovalent-bispecific antibodies (a process called "Fab-arm exchange") (23). S_{228}P mutation of IgG4 can efficiently eliminate fab-arm change. Therefore, the majority of recently approved therapeutic IgG4 antibodies adopt an S_{228}P mutation (24). In IgG2, several disulfide bond isomers (IgG2A, IgG2B, and IgG2A/B) can be formed (25, 26). Many factors such as cell culture conditions or thermal stress contribute to the formation and equilibrium of different isomers (27). *In vivo*, IgG2A isomer can convert to the form of IgG2B (28). Among the three isomers, IgG2B has the most compact structure (26). In addition, as compared to the form of IgG2A, the IgG2B conformation imparts super-agonistic properties to immunostimulatory antibodies, such as anti-CD40 antibodies (29). The feature of IgG2 isomer transformation is FcγR-independent and its activity has been demonstrated for IgG2 CD40 mAb in the clinical trial CP870-893 (29).

TABLE 1 | Binding activities of human FcγR to IgG isotypes and resulting effector functions.

FcγR	Variants	IgG1		IgG2		IgG4	
		Affinity[a]	Effector functions	Affinity	Effector functions	Affinity	Effector functions
I	NA	High	ADCP	None	None	High	ADCP, Cytokine release
IIa	H_{131}	Medium	ADCP	Medium	Myeloid cell-induced ADCC[b]	Low	Receptor clustering[c]
	R_{131}	Low		Low		Low	
IIb	I_{232}[d]	Low	Clearance of IC, Immuno-suppression	None	None	Low	Clearance of IC, Immuno-suppression
	T_{232}[d]						
IIIa	V_{158}	Medium	ADCC	Low	None	Low	None
	F_{158}	Low		None		None	

[a] *Affinity values are based on IC binding to FcγR, adapted from Bruhns et al. (13).*
[b] *Based on Arce Vargas et al. (15).*
[c] *Based on Oberst et al. (16).*
[d] *The T_{232} variant is less potent in inhibitory activity than the I_{232} variant (17). However, the I_{232}T mutation leads to significantly better phagocytosis (18).*

In mice, IgG2A functionally resembles human IgG1, whereas mouse IgG1 is considered the closest functional equivalent of human IgG4. The D_{265}A mutation can further reduce the affinity of mouse IgG1 for the Fc receptor, leading to a "silent Fc" and antibodies harboring this mutation have been widely used in mouse models to evaluate the effects of FcγR-binding on *in vivo* therapeutic efficacy (30–32).

Based on the differences in structure, function, and affinity for IgG binding, FcγRs are classified into three major groups: FcγRI, FcγRII (FcγRIIa and FcγRIIb) and FcγRIII (FcγRIIIa and FcγRIIIb) (13). Among them, FcγRI, FcγRIIa, and FcγRIIIa are activating receptors containing the signal transduction motif, immunoreceptor tyrosine-based activation motif (ITAM), in the γ subunit of FcγRI and FcγRIIIa, or in the cytoplasmic tail of FcγRIIa (14). In contrast, FcγRIIb is an inhibitory receptor. Cross-linking of FcγRIIb leads to the phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) and inhibitory signaling transduction (33).

FcγRI

FcγRI is a high-affinity Fc receptor for both the monomeric IgG and immune complex (IC) (13). The affinities of FcγRI

to IgG1 or IgG4 are similar (K_D of 1–10 nM). In contrast, FcγRI has no binding to IgG2. FcγRI is mainly expressed on monocytes/macrophages, dendritic cells (DCs), and activated neutrophils. One of the major functions of FcγRI is to activate myeloid cells to phagocytose IgG1 and IgG-bound target cells *via* ADCP (34). Due to high-affinity binding of FcγRI to monomeric IgG and high serum concentrations of IgG (∼15 mg/mL), it is believed that most FcγRI is occupied by endogenous IgG (35). However, a recent study has shown that stimulation of myeloid cells with cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), could induce the clustering of FcγRI and increase the binding of FcγRI to ICs (36). Multiple studies have also shown that FcγRI plays an important role in modulating immune responses in autoimmune diseases, inflammation, and antibody therapy (37–39).

FcγRIIA AND FcγRIIIA

Both FcγRIIa and FcγRIIIa are low-affinity FcγRs, which bind weakly to monomeric IgG, but strongly to IC. FcγRIIa and FcγRIIIa receptors are primarily expressed on monocytes/macrophages, dendritic cells, natural killer cells and platelets. FcγR polymorphisms exist in FcγRIIa and FcγRIIIa receptors, resulting in two isoforms of each receptor: H_{131} and R_{131} of FcγRIIa(40), V_{158} and F_{158} of FcγRIIIa (41), respectively. FcγRIIa-H_{131} variant is considered a high responder as compared to R_{131} variant (low responder) due to a higher affinity for IgG1 and increased effector functions (such as phagocytosis) (13, 22). Similar to FcγRI, FcγRIIa is one of the major phagocytic FcγRs that mediates ADCP. In human, FcγRIIIa is the primary receptor for NK- and macrophage-mediated ADCC. FcγRIIIa-V_{158} variant (high responder) has a higher affinity for IgG1 and can also interact with IgG4 (13). Functionally, IgG-induced NK cell activity is increased in FcγRIIIA-V/V_{158} homozygotes compared with FcγRIIIA-F/F_{158} individuals (42).

FcγRIIB

FcγRIIb is expressed on many types of immune cells including B cells, DCs, monocytes/macrophages, mast cells and basophils (33). In addition, FcγRIIb was found to be expressed on liver sinusoidal endothelial cells (LSEC) and plays an important role in IC clearance (43). On B cells, FcγRIIb functions as a primary inhibitory FcγR to suppress B cell activation and antigen internalization after binding to the immune complex (33). FcγRIIb also inhibits the type I interferon production by DCs. The binding affinities of monomeric IgG to FcγRIIb are extremely low ($K_A \approx 2 \times 10^5 M^{-1}$), whereas the affinities of IC to FcγRIIb are significantly higher (13). Despite the critical roles of FcγRIIb in the negative regulation of immune responses, several studies have shown that FcγRIIb is required for the induction of efficient anti-tumor activity by agonistic anti-TNF receptor superfamily-antibody therapeutics such as anti-CD40 antibodies (44, 45). The overall binding features of human FcγR to IgG isotypes are summarized in **Table 1**.

MOUSE FcγRIV

In addition to the FcγRs described above, in mice, there is a unique FcγR (i.e., FcγRIV), whose expression is restricted to myeloid lineage cells (46). FcγRIV bind to mouse IgG2a and IgG2b with intermediate affinity and plays critical roles in IgG2a- and IgG2b-mediated *in vivo* efficacy (46, 47). Mouse FcγRIV is functionally similar to human FcγRIIIa, but not expressed on natural killer cells (47). In a mouse model, anti-CTLA-4 antibody-mediated depletion of Tregs is largely dependent on FcγRIV (10).

Fc ENGINEERING TO REDUCE OR ELIMINATE FcγR BINDING

Several modifications to IgG can directly affect their binding to FcγRs. The $N_{297}A$ mutation was the first mutation to be described with significantly reduced FcγR-binding (48). It was later demonstrated that mutations of residues 234 and 235 in the lower hinge region (EU numbering system) to alanine could also lead to significantly reduced FcγR-binding; the $L_{234}A/L_{235}A$ double mutation on the human IgG1 backbone is also known as the "LALA" mutation (49). In addition, hybrid antibody isotype IgG2m4, which is based on the IgG2 with four key amino acid residue changes derived from IgG4 ($H_{268}Q$, $V_{309}L$, $A_{330}S$, and $P_{331}S$), has been shown to have significantly reduced FcγR binding (50).

IMMUNE CHECKPOINT MOLECULES AND THEIR THERAPEUTIC ANTIBODIES

CTLA-4

CTLA-4 (cytotoxic T-lymphocyte-4, or CD152) is a member of the Ig superfamily, which plays a critical role in inhibiting T-cell immunity (51). The ligands are the B7 family members, CD80 (B7-1) and CD86 (B7-2). As a CTLA-4-related protein, CD28 is constitutively expressed on naïve T cells and enhances T-cell activation when engaged by B7-1/2 on antigen-presenting cells (APC) (52, 53). In contrast, CTLA-4 surface expression increases in a day or two after T cell activation (51, 52). CTLA-4 is also highly expressed on Tregs and plays an important role in the homeostasis and suppressive functions of Tregs (54). There is no known canonical immunoreceptor tyrosine-based inhibitory (ITIM) motif in the cytoplasmic tail of CTLA-4 (55). The exact signaling pathway of CTLA-4 upon engagement with its ligands still remains largely unknown. Accumulating evidence suggested that CTLA-4 primarily exerted its inhibitory functions by competing off CD28 binding to CD80 and/or CD86, due to the higher affinity of CTLA4 to CD80 or CD86 (55). In addition, CTLA-4 has been shown to down-regulate CD80 and CD86 on APC, thus inhibiting CD28-mediated co-stimulation (54).

In mouse tumor models (melanoma and colorectal cancer), several groups have clearly shown that surrogate anti-CTLA-4 antibody-mediated anti-tumor efficacy is dependent on Fc

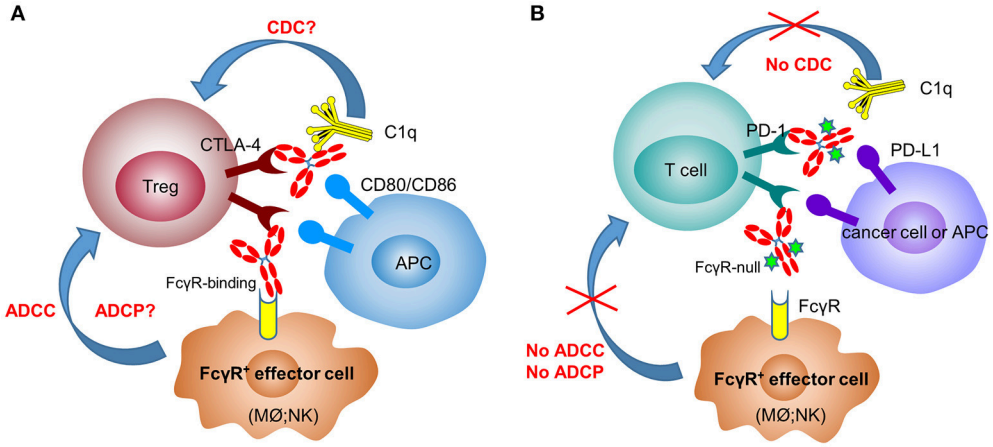

FIGURE 1 | Anti-CTLA-4 and anti-PD-1 therapeutic antibodies have differential FcγR-binding requirement for optimal activity. In the mechanisms of action of anti-CTLA-4 antibodies **(A)**, depletion of Tregs after engaging $FcγR^+$ effector cells [macrophages (Mϕ) and NK cells] plays a critical role in their efficacy. In contrast, anti-PD-1 antibodies need to have the Fc-mediated effector functions (ADCC, ADCP, and CDC) removed to avoid the killing of $PD\text{-}1^+$ T cells by $FcγR^+$ effector cells **(B)**.

effector functions and correlate with depletion of tumor-infiltrating Tregs (10, 30, 56) (**Figure 1A**). In 2011, the FDA approved the first anti-CTLA-4 antibody, ipilimumab (IgG1 wild-type), for the treatment of melanoma. Furthermore, the combination of PD-1 blockade with ipilimumab demonstrated increased, durable anti-tumor activity in renal cell carcinoma and non-small cell lung cancer (NSCLC) (57, 58). Interestingly, anti-CTLA-4 clones, which lose the ability to block the B7-CTLA-4 interaction, remain fully active in inducing tumor rejection, suggesting that other mechanisms are involved in anti-CTLA-4 antibody-mediated anti-tumor efficacy besides the blocking of B7-CTLA-4 (59). In an *ex-vivo* assay, melanoma patient-derived non-classical monocytes could kill Tregs via ADCC (60). In addition, patients who responded to ipilimumab tended to have a higher percentage of $CD14^+CD16^+$ monocytes in the periphery. Using human FcγR-transgenic mice, Arce Vargas et al. clearly demonstrated that antibodies with isotypes equivalent to ipilimumab increased the $CD8^+$ to Treg ratio by depleting intra-tumoral Tregs to promote tumor rejection (15). Furthermore, a response to ipilimumab in melanoma patients is associated with a high-affinity FcγRIIIa ($CD16\text{-}V_{158}$) polymorphism. A second anti-CTLA-4 mAb, tremelimumab, is a human IgG2 isotype with minimal FcγRIIIa-mediated ADCC effects (61). However, anti-mouse CTLA-4 antibody with human IgG2 isotype could also deplete Tregs in human FcγR-transgenic mice in a FcγRIIa-dependent manner (15). Despite the convincing data from mouse models, there has not been direct evidence indicating that anti-CTLA-4 immunotherapy could efficiently deplete Tregs in human cancers (62, 63).

PD-1/PD-L1

In recent years, immune therapy targeting the PD-1/PD-L1 pathway has become a backbone clinical strategy for cancer treatment. Programmed cell death 1 (PD-1) is an inhibitory immune modulatory receptor (64–66). It is inducibly expressed on activated T, NK, and B lymphocytes (67), macrophages, DCs (68), and monocytes (69) as an immune suppressor for both adaptive and innate immune responses. PD-1 is highly expressed on tumor-specific T cells. Engagement of PD-1 by its ligands, PD-L1 (70) or PD-L2 (71, 72) leads to the exhaustion of T cell function and immune tolerance in the tumor microenvironment. Blockade of PD-1 pathway has been shown to restore the function of "exhausted" T cells, resulting in significant anti-tumor activity (70, 73). To date, five PD-1 antibodies have been approved and many others are under development for the treatment of a broad spectrum of cancers (**Table 2**). Most of these anti-PD-1 antibodies are of IgG4 isotype with the $S_{228}P$ mutation (IgG4 $S_{228}P$), which has similar effector-binding properties as the natural IgG4 with reduced ADCC and "null" CDC, but still retaining high affinity to FcγRI and binding to FcγRIIb. In the MC38 mouse model, Dahan et al. reported that engagement of FcγRs reduced the anti-tumor activity of an anti-PD-1 antibody by eliminating $CD8^+$ tumor-infiltrating lymphocytes (TILs) via ADCC in a FcγRI-dependent manner (9). In addition, engagement of FcγRIIb by an anti-PD-1 antibody could also decrease its anti-tumor activities. Arlauckas et al. demonstrated that anti-PD-1 antibodies can be captured from the T-cell surface by FcγR-bearing macrophages. The blockade of FcγRs could thus prolong the binding of the anti-PD-1 antibody to $CD8^+$ TILs and enhance the anti-tumor activity *in vivo* (74). A preclinical study by our group also suggested that FcγRI binding had a negative impact on the anti-tumor activity of anti-PD-1 antibodies in a humanized xenograft model. The binding could induce $FcγRI^+$ macrophages to phagocytose $PD\text{-}1^+$ T cells via ADCP and reverse the function of an anti-PD-1 antibody from blocking to activating (37). Recently, several published research papers documented the phenomenon that the

TABLE 2 | Select PD-1 and PD-L1 antibodies under development for cancer treatment.

Target	Company	mAb	Clinical stages	IgG isotype or mutant with effector function nullified
PD-1	Bristol-Myers Squibb	Nivolumab	Approved	IgG4 $S_{228}P$
PD-1	Merck	Pembrolizumab	Approved	IgG4 $S_{228}P$
PD-1	Regeneron/Sanofi	Cemiplimab	Approved	IgG4 $S_{228}P$
PD-1	Novartis	Spartalizumab	Phase 3	IgG4 $S_{228}P$
PD-1	BeiGene	Tislelizumab	Phase 3	IgG4mut, FcγR null
PD-1	Junshi	JS001	Approved	IgG4 $S_{228}P$
PD-1	Hengrui	Camrelizumab	Phase 3	IgG4 $S_{228}P$
PD-1	Innovent	Sintilimab	Approved	IgG4 $S_{228}P$
PD-L1	Roche	Atezolizumab	Approved	IgG1mut, FcγR null
PD-L1	AstraZeneca	Durvalumab	Approved	IgG1mut, FcγR null
PD-L1	Merck KGaA/Pfizer	Avelumab	Approved	IgG1

hyperprogression frequencies of certain cancer types treated with FDA-approved anti-PD-1 antibodies were substantially higher than the control chemotherapy group (75–77). Lo Russo et al. linked the interaction between the anti-PD-1 antibody and FcγR$^+$ macrophages to the hyperprogression in NSCLC during PD-1 blockade therapy (78). Based on these observations, an anti-PD-1 antibody with pure blocking activity would be more desirable, since an anti-PD-1 antibody with FcγR-binding activity can mediate cross-linking between PD-1$^+$ T-cells and FcγR$^+$ macrophages, induce the depletion of PD-1$^+$ T effector cells, and thus compromise the T-cell activity of tumor growth inhibition (9, 37, 74) (**Figure 1B**).

Programmed death ligand 1 (PD-L1) is constitutively expressed by immune cells of myeloid lineages (79) and the cells at immune-privileged sites (80, 81). It is also inducibly expressed on T, NK and B lymphocytes, epithelial and endothelial cells upon stimulation by pro-inflammatory factors, such as IFN-γ and TNF-α (82). PD-L1 is the main ligand of PD-1, and the PD-L1/PD-1 axis is the major controller of the peripheral immune tolerance (65). In tumors, PD-L1 is expressed on both tumor cells (83) and tumor-infiltrating immune cells and can suppress anti-tumor immunity independently (84). Unlike anti-PD-1 antibodies, the three approved PD-L1 antibodies have differentiated FcγR-binding properties (**Table 2**). Atezolizumab and durvalumab are designed to eliminate FcγR-binding and effector functions (85, 86), while avelumab retains intact Fc functions (87). Recent preclinical data suggested that the engagement of FcγRs could augment the anti-tumor activity of anti-PD-L1 antibodies via the ADCC effect against the PD-L1$^+$ immune suppressive myeloid cells (88) or tumor cells (89). However, it is also speculated that the effector function could be detrimental to the anti-tumor immunity due to the depletion of PD-L1$^+$ APC cells and T effector cells. To understand the role of FcγR-binding on anti-PD-L1 anti-tumor efficacy, future studies are needed to elucidate the expression of PD-L1 in the tumor microenvironment and the effect of anti-PD-L1 antibody treatment.

TIM-3

TIM-3 (T cell immunoglobulin and mucin-domain containing-3, also known as HAVCR2) is a member of the T-cell immunoglobulin- and mucin-domain-containing family that plays an important role in promoting T-cell exhaustion in both chronic viral infections and tumor escape from immune surveillance (90, 91). It is primarily expressed on immune cells, such as T cells, NK cells, DCs, and monocytes/macrophages (92). When expressed on effector T cells, activation of TIM-3 has been shown to reduce cytokine production, T-cell proliferation, and cytotoxicity, all of which could be rescued by TIM-3 blocking antibodies (93, 94). TIM-3 is also expressed on FoxP3$^+$ Treg cells, especially in human tumor tissues, and is correlated with poor clinical parameters (95, 96).

Four TIM-3 ligands have been identified, which include PtdSer, Gal-9, carcinoembryonic antigen-related cell adhesion molecule 1, and high mobility group box 1 (97). To date, the detailed mechanisms of TIM-3 signaling remain unclear. Upregulation of TIM-3 expression in TILs, macrophages, and tumor cells has been reported in many types of cancers (98–101). Increased expression of TIM-3 in those cancers is associated with a poor prognosis and/or patient survival.

Following PD-1 antibody blockade, TIM-3 expression has been shown to be upregulated on TILs from both patient samples and animal models, resulting in "adaptive resistance" to anti-PD-1 treatment (102–104). Blockade of the TIM-3 receptor alone or in combination with PD-1/PD-L1 blockade has been shown both *in vitro* and *in vivo* to rescue functionally "exhausted" T cells (3, 93, 105).

In pre-clinical mouse models of colorectal cancer (MC38 and CT26), the effects of "silent" Fc vs. "competent" Fc on TIM-3 antibody-mediated anti-tumor activity with or without anti-PD-1 antibody treatment were evaluated by several groups (106, 107). The results showed that the combination of "Fc-silent" TIM-3 Ab with PD-1 Ab led to significantly more synergistic tumor-inhibitory effects than the one with "competent" Fc, while TIM-3 blocking Ab monotherapy demonstrated marginal anti-tumor efficacy. The exact mechanisms of Fc effector functions (ADCC and/or ADCP) in the negative regulation of anti-TIM-3 antibody-mediated anti-tumor efficacy remain unknown.

To date, the first-in-human phase 1/2 clinical trials have been initiated for four anti-TIM-3 antibodies: TSR-022 (NCT02817633), MBG543 (NCT02608268), BMS-986258 (NCT03446040), and LY3321367 (NCT03099109). TESARO recently released the clinical data of TSR-022, in monotherapy or in combination with an anti-PD-1 antibody (TSR-042) in patients who progressed following anti-PD-1 treatment (108). The results showed that the combination of TSR-022 and TSR-042 (500 mg) was generally well-tolerated in both NSCLC and melanoma patients, and clinical activities have been observed in the combination therapy, especially at a high dose of TSR-022 (300 mg) with an objective response rate (ORR) of 15% (3/20) and 40% stable disease (8/20) (108).

LAG-3

LAG-3 (Lymphocyte activation gene-3, or CD223) is a member of the immunoglobulin superfamily (IgSF) (109). The immune-regulatory roles of LAG-3 were demonstrated in LAG-3 knockout mice, in which increased susceptibility to autoimmune diseases was observed (110, 111). LAG-3 is primarily expressed on activated T, natural killer (NK), and plasmacytoid dendritic cells (pDC), but not on resting T cells (109, 112). In addition, LAG-3 expression on Tregs is positively correlated with their immune-suppressive activity (113). Sequence homology analysis revealed that LAG-3 is structurally related to CD4, but with higher affinity (60 nM) to MHC class II (MHC-II) molecules, thus inhibiting CD4-MHC-II interaction and negatively regulating T-cell receptor (TCR) signaling (109, 114). In addition, LAG-3 can exert negative regulation of $CD8^+$ T cells via $CD4^+$ T cell-dependent and/or independent manners (115, 116). Similar to PD-1, LAG-3 is expressed on tumor-infiltrating lymphocytes (TILs), but to a less extent. Besides MHC-II molecules, LAG-3 has been shown to bind to galectin-3 (Gal-3) and LSECtin (115, 117). The exact biological function of these two ligands binding to LAG-3 remains unknown. Recently, fibrinogen-like protein 1 (FGL1) has been identified as a novel high-affinity ligand for LAG-3 (118). *In vitro*, FGL1 could induce T-cell inhibition in a LAG-3-dependent manner. In the MC38 colorectal cancer model, ablation of FGL1-LAG-3 interaction with either anti-FGL1 or anti-LAG-3 blocking antibodies inhibits tumor growth.

In mouse tumor models (Sa1N fibrosarcoma, MC38 colorectal cancer, and MBT-2 bladder cancer), dual blockade of LAG-3 and PD-1 receptors with blocking antibodies has shown to significantly improve the anti-tumor activity than either antibody alone (111, 119). In a study by Jun et al., a pair of anti-mouse LAG-3 surrogate antibodies with IgG1 (D265A) [anti-mLAG-3 IgG1(D265A)] or IgG2a (anti-mLAG-3 IgG2a) isotypes were generated based on a commercial clone (C9B7W). Comparative study of these two antibodies either alone or in combination with anti-mouse PD-1 antibody in the CT26 mouse colorectal cancer model showed that anti-mouse LAG-3 antibody with minimal Fc effector functions [IgG1 (D265A)] had anti-tumor efficacy, and the one with effector function (IgG2a) had no apparent tumor inhibitory effect (120). In addition, when combined with PD-1 blocking antibody, anti-mLAG-3 IgG1 (D265A) showed significantly synergistic anti-tumor effects, whereas anti-mLAG-3 IgG2a with intact effector function in combination with an anti-mouse PD-1 antibody was less efficacious than anti-mouse PD-1 alone, suggesting that the effector function of LAG-3 antibody might interfere with anti-mouse PD-1 mediated efficacy. The anti-tumor efficacy of anti-mouse LAG-3 antibodies without effector functions was also observed by other groups (119, 121, 122).

As of now, there are six LAG-3 antibodies being evaluated in clinical trials. All these LAG-3 antibodies have Fc with either reduced or "null" effector functions. Preliminary data showed that combining anti-LAG-3 therapy (BMS-986016) with nivolumab in melanoma patients refractory to PD-1/PD-L1 treatment could help patients overcome resistance and restore T-cell function with an ORR up to 18%, especially in patients with high LAG-3 expression ($\geq$1%)(123).

TIGIT

TIGIT (T cell immunoglobulin and ITIM domain, also known as WUCAM or Vstm3) is a member of the CD28 family of proteins that play an important role in inhibiting T- and NK cell-mediated functional activities in anti-tumor immunity (124–126). TIGIT is mainly expressed on T and NK cells. T cells in the tumor microenvironment (3) often co-express TIGIT with other "checkpoint" inhibitory immune receptors, such as PD-1, LAG-3, and TIM-3 (93, 127).

Two TIGIT ligands, CD155 (PVR) and CD112 (PVRL2, nectin-2), have been identified; they are primarily expressed on APCs (such as dendritic cells and macrophages) and tumor cells (125, 126, 128, 129). The binding affinity of TIGIT to CD155 (*Kd*: ~1nM) is much higher than to CD112. Whether the TIGIT: CD112 interaction is functionally relevant in mediating inhibitory signals is yet to be determined. High-affinity binding of TIGIT to CD155 could compete with another co-stimulatory receptor, CD226 (DNAM-1), which binds to the same ligands with lower affinity (*Kd*: ~100nM) and delivers a positive signal (130), therefore reducing T- or NK-activation. In addition, the interaction between TIGIT and PVR on dendritic cells (DCs) could deliver a "reverse signaling" in DCs, leading to reduced DC activity and T-cell activation (126). TIGIT expression on Tregs has been associated with a highly immune-suppressive phenotype in tumor tissue and TIGIT signaling in Tregs may favor Treg stability (131, 132).

Blockade of the TIGIT receptor alone or in combination with PD-1/PD-L1 blockade could rescue functionally "exhausted" T cells both *in vitro* and *in vivo* (133, 134). In the CT26 cancer model, Fc with effector functions is critical for TIGIT antibody-mediated anti-tumor activity (11, 135). The TIGIT antibody with wild-type (WT) human IgG1 Fc (EOS884448) has been shown to be capable of preferentially depleting Treg cells *in vitro* (11). The authors demonstrated that the surrogate mouse TIGIT antibody of the mIgG2a isotype has potent anti-tumor activity either as monotherapy or in combination with a PD-1 antibody. In contrast, the mouse anti-TIGIT antibody with Fc devoid of effector functions did not show any of the anti-tumor efficacies, indicating that Fc-mediated effector functions are required for TIGIT antibody-mediated anti-tumor effects. In addition, the observed efficacy was associated with increased activity of effector T cells ($CD8^+$ and $CD4^+$) and also with Treg depletion within the TME. Argast et al. also observed that effector functions were critical for TIGIT antibody-induced *in vivo* efficacy (135).

To date, there are six TIGIT antibodies (see **Table 3**) in clinical trials, with different IgG isotypes or mutant forms. The most advanced, MTIG7192 (NCT03563716), is in a phase 2 trial in combination with the anti-PD-L1 antibody atezolizumab for treatment of advanced NSCLC. How the effector functions affect clinical activities remains to be seen.

TABLE 3 | Anti-TIGIT in clinical trials.

Company	mAb	Clinical stages	IgG Isotype and Fc effector functions
Genentech	MTIG7192	Phase 2	IgG1
Merck Sharp & Dohme	MK-7684	Phase 2	IgG1
Bristol-Myers Squibb	BMS-986207	Phase 1/2	IgG1mut, FcγR null
Oncomed	OMP-313M32	Phase 1	IgG1
Arcus	AB-154	Phase 1	IgG4 $S_{228}P$
Potenza	ASP8374	Phase 1	IgG1mut, FcγR null

CD73

CD73 (also known as 5'-ecto-nucleotidase, or NT5E) is a glycosylphosphatidylinositol (136) anchored cell surface protein, which has both enzymatic and non-enzymatic functions (137). As a nucleotidase, it catalyzes the extracellular dephosphorylation of adenosine monophosphate (AMP) to adenosine. Adenosine is believed to be an immunosuppressive molecule inhibiting $CD8^+$ T cells, NK cells, and dendritic cells, while promoting the proliferation of immunosuppressive cells (138, 139). In some cases, CD73 can be shed from the cell surface with retained enzymatic activity (140). Expression of CD73 varies on normal tissues but remains at constitutively high levels on many types of cancer cells. High CD73 expression has been shown to be correlated with unfavorable clinical outcomes (141–147), which is consistent with the immunosuppressive role of adenosine.

Three CD73 blocking antibodies have been entered into clinical trials (i.e., BMS-986179, CPI-006, and MEDI9447). Compared with small-molecule inhibitors, anti-CD73 mAbs offer the possibility of directly targeting both enzymatic and non-enzymatic CD73 pathways (148). *In vitro* data showed that MEDI9447 (human IgG1 variant) could inhibit the enzymatic activity of both soluble- and membrane-bound CD73 through prevention of the conformational transition of CD73 to an active state, and could induce internalization of membrane-bound CD73, and restore T-cell proliferation from the inhibition by AMP (149, 150). In a mouse model, MEDI9447 monotherapy showed significant anti-tumor efficacy, which was further increased when combined with a PD-1 antibody (150). In the Fc region of MEDI9447, triple mutations ($L_{234}F/L_{235}E/P_{331}S$) were introduced to eliminate its binding to FcγRs (Including FcγRI, FcγRIIa, and FcγRIIIa) and C1q (150, 151). Similarly, CPI-006 from Corvus is also an IgG1 isotype with a "silent" Fc. It could fully block the production of adenosine by inhibiting the enzymatic activity of CD73 (IC50, 17nM) without internalization, while also activate B cells independent of adenosine reduction (152).

Another anti-CD73 antibody, BMS-986179, is an IgG2/IgG1 hybrid with a "null" effector function. BMS-986179 could not only inhibit CD73 enzymatic function but also induce rapid, near-complete internalization (153). The disulfide bond isomerization of IgG2 is thought to be the major mechanism for BMS-986179-induced CD73 efficient clustering and internalization. Results from mouse models indicated that the combination of PD-1 blockade and a surrogate anti-mouse-CD73 antibody treatment resulted in more enhanced anti-tumor efficacy than either treatment alone (153). In a phase 1/2a study (NCT02754141), 59 patients with advanced solid tumors were treated either alone with BMS-986179 or in combination with nivolumab. Preliminary results showed that both the monotherapy of BMS-986179 and the combination were well-tolerated and clinical activities were observed with 7 partial responses and 10 stable diseases (154).

VISTA

VISTA (V-domain Ig-containing Suppressor of T cell Activation,also known as B7-H5, B7H5, C10orf54, DD1alpha, GI24, PD-1H, PP2135, SISP1) is a type I transmembrane protein with a single extracellular IgV domain, functioning as a negative regulator of T-cell immunity. It is predominantly expressed on hematopoietic cells, at the highest level on myeloid cells and at lower levels on T cells (155). *In vitro* studies indicated that not only could VISTA-Ig inhibit T-cell activation and proliferation, but it could also induce Treg differentiation (155). The receptor for VISTA remains unknown. Results from murine models suggested that VISTA and PD-1 suppressed T-cell function in a synergistic manner, providing the possibility of combined therapy targeting both VISTA and PD-1 to enhance anti-tumor immunity (156).

To date, JNJ-61610588, a fully human IgG1 antibody (with wild-type Fc) is the only anti-VISTA monoclonal antibody in a clinical trial (NCT02671955). A preliminary study showed that JNJ-61610588 could induce monocytes and T-cell activation, as well as T-cell proliferation *in vitro* (12). Interestingly, active Fc and Fc receptor crosslinking is required for the efficacy, since neither the silent Fc version of VSTB140, with an IgG2 sigma constant region, nor the Fc blocking of JNJ-61610588 exhibited activity. Consistent with *in vitro* findings, the anti-tumor activity of JNJ-61610588 in mouse tumor models was observed. The exact mechanisms and clinical evidence remain to be seen.

B7-H3

B7-H3 (Human B7 homolog 3, also known as CD276) is a member of the B7 family of immune proteins. The majority of studies suggest that B7-H3 is an immune checkpoint molecule (157–159), although it was initially characterized as a co-stimulatory molecule for T-cell activation and IFNγ production (160). The B7-H3 receptor expressed on T cells remains to be identified (161). B7-H3 has limited expression on normal tissues but is preferentially expressed on a wide spectrum of cancer cells and tumor vasculature, which is associated with poor outcomes in multiple cancers (162–168).

MGA271 (or enoblituzumab), is an Fc-enhanced humanized IgG1 anti-B7-H3 antibody developed by MacroGenics. Mutations were introduced in the IgG1 Fc domain to increase its affinity to FcγRIIIa but decrease the affinity to FcγRIIb (169). Enhanced ADCC against a wide arrange of B7-H3 positive tumor cell lines (including prostate, lung, breast, colon, bladder, renal cancers and melanoma) was observed across all the

donors with different FcγRIIIa polymorphisms (low-affinity 158F homozygous, high-affinity 158V homozygous, and 158F/V heterozygous). Consistent with *in vitro* data, greater anti-tumor efficacy was observed in the group with MGA271 than the one with wildtype IgG1 Fc in human FcγRIIIa-158F-transgenic mice (170). Initial evidence of anti-tumor activity was observed in a clinical trial with MGA271, with no dose-limiting toxicities or severe immune-related side effects (171).

CONCLUDING REMARKS

In this review, we have summarized recent advances in the study of FcγR-binding on checkpoint antibody therapy. For targets such as CTLA-4, multiple studies indicated the critical role of competent IgG1-Fc for anti-CTLA-4 antibody-mediated intratumoral depletion of Tregs *via* ADCC (10, 15). This MOA may largely be attributed to the preferential surface expression of CTLA-4 on Tregs and the presence of significant numbers of $CD16^+$ macrophages inside tumors (15). In mouse models, anti-CTLA-4 mAbs do not block CTLA-4-B7 interaction, yet they remain active in anti-tumor efficacy, suggesting that intratumoral depletion of Tregs by anti-CTLA-4 antibodies might be the primary MOA (172). A similar phenomenon was observed for TIGIT or VISTA in mouse models, in which their antibody-elicited anti-tumor efficacy is mainly dependent on Fc-mediated effector functions (11).

So far, five approved anti-PD-1 mAbs (nivolumab, pembrolizumab, and cemiplimab) are of human IgG4 isotype. The choice was made primarily based on the fact that the affinity of IgG4 to FcγRIIIa is very low, inducing little ADCC (13). However, IgG4 binds to FcγRI with high affinity, which can negatively impact the efficacy of PD-1 therapy (9, 37). Moreover, IgG4 can also bind to FcγRIIb, leading to reduced anti-tumor efficacy, likely through the induction of a more immunosuppressive environment (9, 78). Therefore, an IgG variant of the anti-PD-1 antibody with null FcγR-binding is expected to be the optimal candidate for therapeutic blocking of PD-1 without the unwanted engagement of FcγR pathways. A similar rationale applies to co-inhibitory receptors TIM-3 and LAG-3, in which blocking antibody-mediated anti-tumor efficacy might be compromised when the Fc maintains intact effector functions.

Three PD-L1-targeting mAbs have been approved: atezolizumab, durvalumab (IgG1 variant with null or reduced Fc-FcγR binding), and avelumab (wild-type IgG1, ADCC-enabling) (173). Comparison of clinical activities of these mAbs may provide important insight into the contribution of FcγRs for the anti-PD-L1 treatment of human cancers.

It should be noted that most of the findings in this review about the role of IgG antibody and FcγR binding on immune-oncology therapy were obtained from mouse models (some even in human FcγR-transgenic mice). There are several factors that need to be taken into consideration, including, how well the mouse FcγR expression pattern (including transgenic human FcγRs) mimics the human counterpart, especially in cancer patients, and how different the abundance and distribution of $FcγR^+$ effector cells (e.g., NK cells and macrophages) are in mice vs. in humans in the TME. Studies on the impact of human FcγR polymorphisms (FcγRIIIa-V_{158} vs. F_{158}; FcγRIIa-H_{131} vs. R_{131}) on clinical activity may also shed light on the MOA of immune checkpoint-targeted antibodies (15). In addition, *ex vivo* assays using human tumor samples and targeted antibodies in various settings may provide useful insight into this matter.

In summary, the triggering of effector functions on IgG and FcγR interactions is a complex process; the overall outcome may be dependent on the target expression level, distribution, and abundance of T cells, and the $FcγR^+$ effector cells (NK cells and macrophages) inside tumors. Further investigation through clinical pathology and pharmacology studies is needed to assess the translational applicability of these findings in mouse models to human cancer treatment.

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frontiers in Immunology

REVIEW
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Targeting the Antibody Checkpoints to Enhance Cancer Immunotherapy–Focus on FcγRIIB

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Immunotherapy with therapeutic antibodies has increased survival for patients with hematologic and solid cancers. Still, a significant fraction of patients fails to respond to therapy or acquire resistance. Understanding and overcoming mechanisms of resistance to antibody drugs, and in particular those common to antibody drugs as a class, is therefore highly warranted and holds promise to improve response rates, duration of response and potentially overall survival. Activating and inhibitory Fc gamma receptors (FcγR) are known to coordinately regulate therapeutic activity of tumor direct-targeting antibodies. Similar, but also divergent, roles for FcγRs in controlling efficacy of immune modulatory antibodies e.g., checkpoint inhibitors have been indicated from mouse studies, and were recently implicated in contributing to efficacy in the human clinical setting. Here we discuss evidence and mechanisms by which Fc gamma receptors–the "antibody checkpoints"–regulate antibody-induced antitumor immunity. We further discuss how targeted blockade of the sole known inhibitory antibody checkpoint FcγRIIB may help overcome resistance and boost activity of clinically validated and emerging antibodies in cancer immunotherapy.

Keywords: therapeutic antibody, antibody checkpoint, fc gamma receptor, cancer immunotherapy, drug resistance, tumor microenvironment

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INTRODUCTION

Monoclonal antibody-based therapies have revolutionized cancer treatment improving survival for patients with hematologic and solid cancers. The clinically most successful antibodies exert antitumor activity either by targeting tumor cells directly (direct-targeting antibodies) (1–4), or by targeting and activating immune cells that seek up and kill cancer cells in the tumor microenvironment (immune checkpoint antibodies) (5–13).

While both types of mAb are highly potent with cancer curative potential a significant fraction of patients fail to respond or develop resistance to treatment (14–17). An improved understanding of mechanisms underlying resistance, and in particular those common to antibody drugs as a class–including direct-targeting and immune checkpoint antibodies–is needed for rational development of drugs that could help boost efficacy, and prevent or overcome antibody drug resistance. Given the broad use of antibodies in cancer treatment, such drugs would have the potential to fundamentally improve cancer survival.

FcγR Regulation of Antibody-Induced Immunity–"The Antibody Checkpoints"

The Fc receptors (FcR) are the only receptors of the immune system known to regulate the activity of antibodies as a class (18). FcRs orchestrate antibody-induced effector cell responses and immunity through low affinity, high avidity interactions with aggregated antibody Fc-domains of antibody-coated cells or immune complexes, generated following antibody Fv-binding to target receptors. Because Fc domains are conserved between antibodies of a given subclass e.g., IgA, IgE, IgM, or IgG_1, IgG_2, IgG_3 or IgG_4, FcRs regulate antibody-induced immune responses irrespective of antigen specificity. For this same reason FcRs regulate immune responses induced both by endogenously generated antibodies (e.g., antibodies mounted in response to infection or underlying inflammatory or autoimmune disease) and recombinantly produced therapeutic monoclonal antibodies (18, 19). Of particular relevance for cancer immunotherapy the Fc gamma receptors (FcγR) are known to regulate the activity of Immunoglobulin G type of antibodies (20), the group to which all antibodies approved for cancer therapy belong.

The family of FcγRs share several characteristics with the T cell immune checkpoints in how they regulate effector cell activation and immune responses (**Figure 1**). Recent work by ourselves and others, reviewed in detail below, demonstrate a critical role for this receptor family as concerted regulators of antibody-induced innate and adaptive immunity. Consequently, the FcγRs are therapeutically important immune checkpoints, and since they control immune activity of IgG antibodies as a class, we propose to refer to them as "antibody checkpoints." We will herein use antibody checkpoint and FcγR interchangeably.

Antibody and T Cell Checkpoints–Similarities and Differences

Like the T cell checkpoints the Fc gamma receptors (FcγR) fall into either of two functionally distinct groups, which coordinately regulate immune effector cell activation and ensuing immune responses (**Figure 1**). Activating FcγR, like co-stimulatory T cell checkpoints, promote effector cell activation, and immunity. In contrast, inhibitory FcγR, like the T cell co- inhibitory checkpoints, block cellular activation and down-modulate immune responses. Adding to complexity, antibody checkpoints may–similar to the T cell checkpoints–promote checkpoint receptor extrinsic signaling by facilitating cross-linking and signaling of ligand receptors (21, 22). In case of the antibody checkpoints, this would equate to FcγR-mediated cross-linking of antibody Fv-targeted receptors (**Figure 2**). Depending on ligand receptor function, such signaling may be activating or inhibitory, as has been described for agonistic CD40 and agonistic Fas antibodies, respectively (23–27). FcγR extrinsic signaling may, or may not, contribute to therapeutic efficacy.

The activating and the inhibitory FcγR receptors transmit their signals into FcγR-bearing immune cell via immunoreceptor tyrosine-based activation motifs (ITAM), and immunoreceptor tyrosine-based inhibitory motifs (ITIM), respectively. Specifically, how target cell-bound antibodies modulate immune cell activation is determined by their relative engagement of activating and inhibitory Fcγ receptors. This in turn is determined by the size of the FcγR-engaging immune complex, i.e., the number of antibodies coated onto a target cell (determined by cellular expression levels of antibody targeted receptor), availability of activating and inhibitory Fcγ receptors, and antibody isotype. Different antibody isotypes bind with different affinity to activating and inhibitory Fcγ receptors, resulting in different activating: inhibitory (A:I) ratios, and differential ability to mediate e.g., activating FcγR-dependent target cell deletion (28) or inhibitory FcγR-dependent agonism (23, 24).

As in the T cell checkpoint family, there are several activating antibody checkpoints that individually, and collectively, positively regulate antibody-induced cell activation. In humans, the activating FcγR's are: FcγRI (CD64), FcγRIIa (CD32a), FcγRIIc (CD32c), and FcγRIIIa (CD16a) (29, 30). The GPI-linked FcγRIIIb lacks an intracellular signaling domain and ITAM motifs, but is nevertheless often considered an activating FcγR, since it has been shown to promote neutrophil activation and effector cell mediated target cell killing in response to challenge with antibody-coated target cells (31, 32). The activating mouse FcγRs are: FcγRI, FcγRIII, and FcγRIV (28, 30, 33).

Most Fc gamma receptors bind monomeric IgG with low to intermediate (μM) affinity [as reviewed in detail elsewhere (28, 29, 33)]. Immune complex formation allows for high-avidity binding of multimerized IgG Fc's to the low-affinity FcγRs, which are cross-linked, leading to FcγR-expressing cell activation. In contrast, free circulating IgG has too low affinity to promote stable Fc:FcγR binding, and cannot promote FcγR-cross-linking, or cell activation. How high affinity FcγRs e.g., FcγRI and mouse FcγRIV, which may bind monomeric uncomplexed IgG, sense and trigger activation in response to immune complexes and antibody-coated cells remains a subject of debate. It is however clear that the high affinity FcγRs may critically contribute to therapeutic antibody efficacy and pathology (33, 34).

Multiple isoforms and allelic variants of the individual FcγRs are known, and the affinities of the clinically most significant variants for different human IgG subclasses have been described (29). Of particular significance for cancer immunotherapy, two isoforms of the low and intermediary affinity antibody checkpoints FcγRIIa (H131R) and FcγRIIIa (V158F), which bind IgG and antibody-coated target cells with higher affinity and avidity, have been associated with improved survival of diverse cancer patients in response to antibody-based cancer immunotherapy (35–39). These, and additional polymorphisms of low and intermediary affinity activating and inhibitory FcγRs, which alter affinity for IgG, or modulate FcγR expression levels, are further associated with susceptibility to antibody-mediated chronic inflammatory and autoimmune disease (40). Of further functional consequence, there is extensive gene copy number variation in high and low affinity loci that affect expression levels of individual FcγRs (41–43).

The antibody checkpoints differ from the T cell checkpoints in notable and critical aspects, which have important consequences for the type of immune response induced, and for design

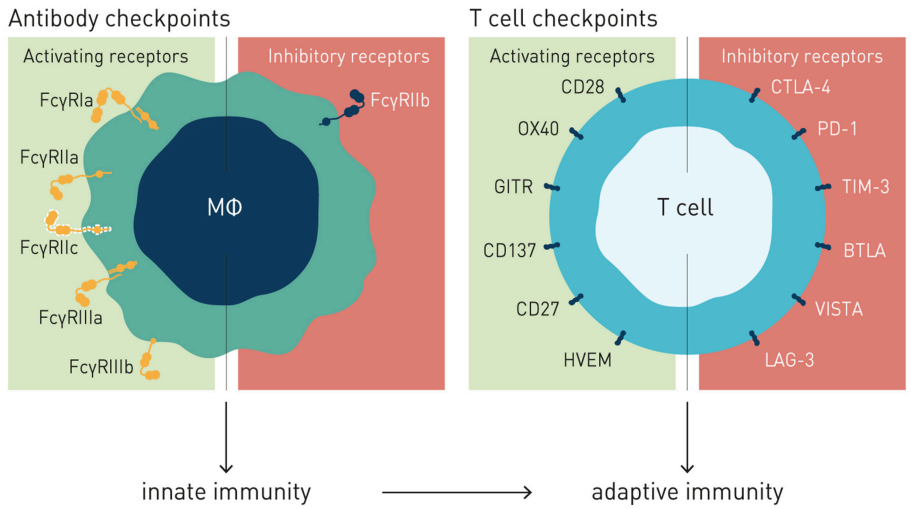


FIGURE 1 | Antibody and T cell checkpoints. Both T cell and antibody checkpoints comprise activating (co-stimulatory) and inhibitory receptors. However, antibody checkpoints are co- expressed only on innate immune cells e.g., macrophages and dendritic cells, and comprise only a single inhibitory member (FcγRIIB).

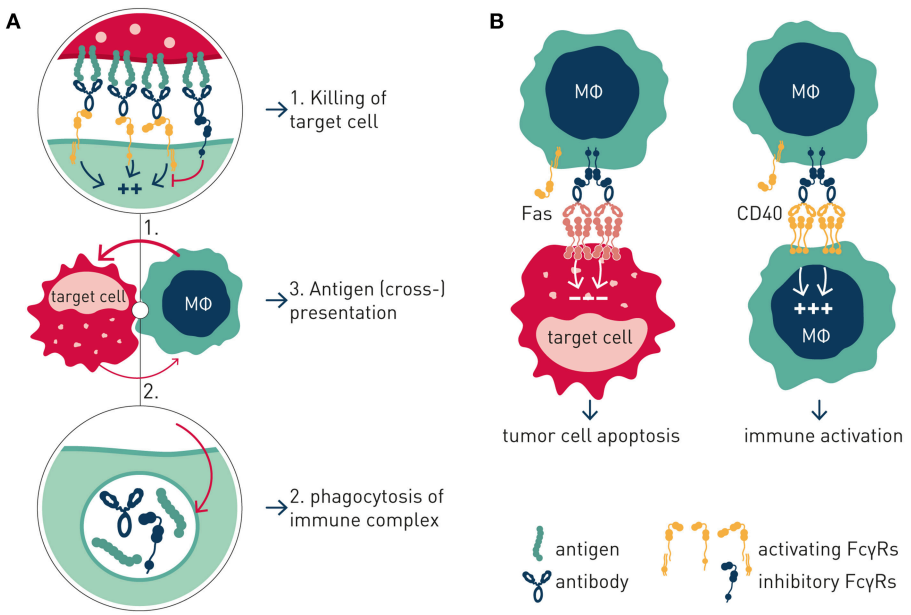


FIGURE 2 | Antibody checkpoint intrinsic and extrinsic signaling. **(A)** Intrinsic signaling. Antibody checkpoints relay aggregated antibody Fc-induced signals into effector cells (MΦ) in a concerted manner through ITAM containing activating (aFcγR) and ITIM-containing inhibitory (iFcγR) Fc gamma receptors. FcγR-expressing cell responses include phagocytosis, immune complex endocytosis, and antigen presentation. **(B)** Extrinsic signaling. Antibody checkpoints promote clustering and signaling induced by antibody targeted receptors in an antibody Fv and Fc co-dependent manner. Cellular responses are determined by the antibody-targeted receptor's function e.g., macrophage co-stimulation or tumor cell apoptosis.

of drugs aimed at harnessing and enhancing FcγR-mediated immunity (**Figure 1**).

Firstly, in contrast to the T cell checkpoints the Fc gamma receptors are not generally expressed on T cells, but principally on cells of the innate immune system, and in a restricted manner on B cells (FcγRIIb) and NK cells (FcγRIIIa and FcγRIIc, the latter in ∼20% of caucasians) (18, 30, 41). In particular cells specialized in MHC class II-restricted antigen presentation, e.g., macrophages and dendritic cells, express both activating and inhibitory FcγRs, enabling fine-tuned regulation of antibody-induced immune responses (28, 44). Consequently, the antibody checkpoints hold the key to unleash antibody-induced immunity first and fore-most through improving innate immune effector mechanisms, e.g., macrophage dependent phagocytosis (ADCP),

and dendritic cell mediated antigen presentation, and cross-presentation (45–51). Triggering and enhancing innate immune activation and robust antigen presentation is known to critically contribute to and underlie robust adaptive T cell-mediated antitumor responses, including those induced by antibodies targeting T cell checkpoints (18, 52–54). Modulation of antibody checkpoints therefore has the potential to improve also adaptive antitumor responses, possibly decreasing the threshold of tumor mutational burden for cancers to respond to antibody-mediated cancer immunotherapy (55). Finally, and in stark contrast to the multiple inhibitory T cell checkpoints described, only a single inhibitory antibody checkpoint–Fc gamma receptor IIB–is known (**Figure 1**).

ANTIBODY CHECKPOINTS DETERMINE ANTI-CANCER ANTIBODY EFFICACY

Cancer Cell Direct-Targeting Antibodies

The CD20-specific antibody rituximab was the first antibody to be approved by the FDA for cancer therapy and is arguably the clinically best validated antibody used in cancer immunotherapy. As such rituximab provides a prime example of a tumor cell direct-targeting antibody that has been exhaustively studied from a mechanism-of-action perspective. While multiple mechanisms, including induction of apoptosis and triggering of complement mediated cell lysis, have been proposed to contribute to and underlie rituximab therapeutic activity (56, 57), the strongest preclinical, and clinical evidence point to Fc gamma receptor dependent mechanisms (58–61).

Independent retrospective studies have established a correlation between one or more activating Fc gamma receptors and clinical efficacy in different types of lymphoma. Patients homozygous for high affinity allelic variants of the activating antibody checkpoints FcγRIIIa or FcγRIIa showed improved responses and survival in response to rituximab therapy compared to patients carrying one or more lower affinity alleles (35, 36). Similar links between response and FcγR-dependent mechanisms have been observed for additional cancer cell direct-targeting antibodies e.g., herceptin (anti-Her2) and cetuximab (anti-EGFR) in breast cancer (38) and colorectal patients, respectively (37, 39). These observations have spurred biotech and pharmaceutical companies to engineer antibodies with improved binding to activating antibody checkpoints. Obinutuzumab, a glycoengineered antibody with improved affinity for FcγRIIIa, was approved for clinical use based on increased overall survival in a head-to-head comparison with rituximab in CLL patients (15). Taken together, these observations demonstrate that antibody checkpoints can determine clinical efficacy of cancer cell direct-targeting antibodies.

Consistent with the well-conserved function of activating and inhibitory antibody checkpoints between mouse and man, similar dependencies between activating FcγRs and cancer cell direct-targeting antibodies have been made in mouse cancer experimental models. Further in keeping with common, ITAM-signaling dependent, functions of the several activating antibody checkpoints, genetic ablation of individual activating FcγRs typically has shown limited effects on *in vivo* therapeutic efficacy compared to ablation of all activatory FcγRs (28, 33, 62).

In stark contrast, genetic deletion of the sole inhibitory antibody checkpoint FcγRIIB fundamentally enhances *in vivo* therapeutic activity of cancer cell direct-targeting antibodies, including those specific for CD20, Her2, and EGFR i.e., clinically validated targets in therapy of hematologic malignancy as well as solid cancer (63). These observations indicate the significant therapeutic potential of targeting the inhibitory antibody checkpoint, and indicate that redundancy needs to be accounted for when seeking to enhance antibody efficacy by modulating activating antibody checkpoints, much as has been observed in targeting of the multiple different T cell checkpoints (6, 14, 64).

Interestingly, and in further support of FcγRIIB being a tractable target in cancer immunotherapy, recent data has demonstrated that this inhibitory antibody checkpoint limits therapeutic antibody efficacy and promotes antibody drug resistance by additional mechanisms distinct from inhibitory signaling in immune effector cells, when expressed on tumor B cells (65) (**Figure 3**). Beers et al. found that FcγRIIB expressed on tumor B cells promoted internalization of rituximab antibody molecules from the tumor B cell surface, increasing antibody consumption and leaving fewer rituximab molecules to engage critical FcγR-dependent effector cell-mediated antitumor activity e.g., ADCP (66). FcγRIIB expression correlated with rituximab internalization across several different lymphoma subtypes studied. Highest and most homogenous expression of FcγRIIB is observed in Chronic Lymphocytic Leukemia (CLL), Mantle cell lymphoma (MCL), and Marginal Zone Lymphoma, although a fraction of Follicular lymphoma (FL) and Diffuse Large B cell Lymphoma show exceptionally high FcγRIIB expression (67, 68). Further consistent with tumor B cell expressed FcγRIIB limiting antibody therapeutic efficacy and promoting antibody resistance, retrospective clinical studies of MCL and FL patients treated with rituximab-containing therapy showed decreased survival of patients with higher FcγRIIB expression on tumor cells (67, 69). Tumor cell expressed FcγRIIB appears to be a general mechanism limiting antibody therapeutic efficacy and promoting antibody drug resistance in the tumor microenvironment. Using a humanized model of treatment refractory B cell leukemia, and the CD52-specific antibody alemtuzumab, Pallasch et al. found that FcγRIIB is highly overexpressed on leukemic tumor cells in such antibody drug-resistant tumor microenvironments, and that shRNA-mediated knock-down of tumor cell FcγRIIB restored responsiveness to therapeutic antibody resulting in animal cure (70). Finally, high expression of FcγRIIB in B cell malignancy may indicate that immunocompetent antibodies to FcγRIIB could have single agent therapeutic activity in this setting (65, 71).

Collectively, these and other observations provided the rationale to develop antagonistic anti-FcγRIIB antibodies that block FcγRIIB-mediated antibody internalization for combination immunotherapy of B cell cancer with direct-targeting antibodies e.g., rituximab (65, 72) (**Figure 3**).

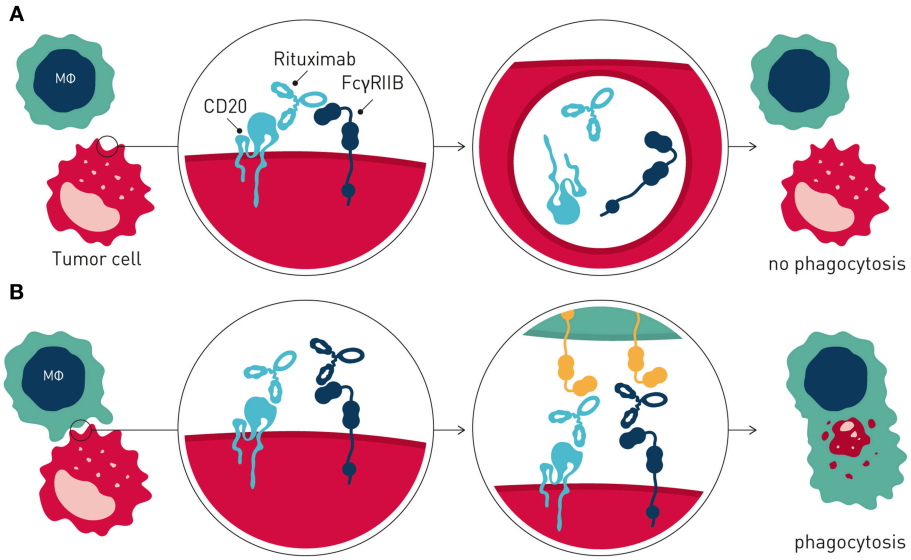


FIGURE 3 | Tumor cell expressed FcγRIIB promotes antibody drug resistance. **(A)** Resistance is mediated by FcγRIIB-mediated removal of antibody molecules from the tumor cell surface through a process of internalization. **(B)** Blocking antibodies to FcγRIIB prevent internalization, leaving greater numbers of therapeutic antibody on the tumor cell surface, promoting immune effector cell-mediated antitumor activity.

Antibodies to Immune Checkpoint Inhibitory Receptors

Antibody targeting of immune inhibitory T cell checkpoints e.g., CTLA-4, PD-1 and PD-L1 has transformed solid cancer therapy shifting focus from cancer cell-direct targeting therapies to immune modulatory drugs, which induce long-term remission and apparent cures albeit in a small fraction of advanced stage cancer patients. Such immune checkpoint-directed therapy has increased overall survival for patients with various cancers, notably including multiple solid cancer types e.g., melanoma, lung, bladder, and head and neck cancer, and are approved by the Food and Drug Administration (14, 73, 74).

While originally thought to act solely via "blocking the brake" on effector T cells (74, 75), recent preclinical and clinical data indicate a critical role for FcγR's in regulating therapeutic efficacy of antibodies to inhibitory T cell checkpoints. Vargas et al. for the first time in human subjects, demonstrated a link between antibody checkpoints, and clinical response to T cell checkpoint targeted antibody therapy (76). Melanoma patients carrying a high affinity allele of the activating FcγRIIIa (V158) showed improved survival in response to treatment with the anti-CTLA-4 antibody ipilimumab compared to patients carrying a lower affinity FcγRIIIa (F158) allele. Interestingly, in the two retrospectively studied cohorts, a prerequisite for response to anti-CTLA-4 antibody therapy was that patients had inflamed tumors i.e., T cells had infiltrated tumors prior to commencing therapy. The observation that antibody checkpoints determine clinical efficacy of ipilimumab was not unexpected, since anti-CTLA-4 antibody therapy in the mouse critically depends on FcγR-mediated deletion of regulatory T cells (77–80), which express CTLA-4 at higher levels compared with effector T cells in the tumor microenvironment (76). Consistent with co-ordinate regulation of anti-CTLA-4 antibody therapeutic efficacy by the antibody checkpoints, in a FcγR-humanized mouse model antibody variants engineered for enhanced binding to activatory FcγR showed enhanced therapeutic activity (76). In contrast, antibody variants with diminished binding to activating FcγR failed to induce protective immunity against cancer.

So, how about the other clinically validated T cell checkpoints? Do antibody checkpoints regulate the activity also of antibodies targeting the PD-1/PD-L1 axis? Evidence from mouse models suggests that indeed they do. Interestingly, however, these data indicate differential FcγR-regulation for anti-PD-1 and anti-PD-L1 antibodies. Dahan et al. reported that anti-PD-L1 antibodies therapeutic efficacy was enhanced with antibody isotypes that preferentially engage activating over inhibitory antibody checkpoints (81). Conversely, anti-PD-1 antibody variants that did not engage FcγRs showed greatest therapeutic activity, and FcγR-engaging antibodies' activity decreased with increasing A:I ratios. Similarly, Pittet and coworkers found that *in vitro* and *in vivo* efficacy of clinically approved anti-PD-1 antibodies nivolumab and pembrolizumab, and a murine surrogate antibody variant with claimed similar engagement of mouse FcγR compared to these mAb, was compromised by FcγR-engagement (82). Deglycosylation of antibodies with EndoS rendering them incapable of engaging FcγRs, or antibody-mediated FcγR-blockade, significantly improved anti-PD-1 antibody therapeutic activity. This demonstrates that FcγRs negatively regulate anti-PD-1 antibody efficacy. Further studies are needed to dissect the relative importance of activating vs. inhibitory antibody checkpoints in regulating anti-PD-1/PD-L1 antibodies' therapeutic activity.

Antibodies to Immune Checkpoint Co-stimulatory Receptors

The power of treating cancer by engaging patient's own immune defense mechanisms through immunotherapy with antibodies to the co-inhibitory T cell checkpoints, has prompted the question of whether targeting also co-stimulatory immune checkpoints e.g., 4-1BB, OX40, CD40, and GITR can translate into similarly efficacious and perhaps complementary pathways of anti-cancer immunity?

Preclinical and limited clinical data has indicated both single agent activity of antibodies to co-stimulatory immune checkpoints and complementary effects following combination with checkpoint blocking antibodies e.g., anti-PD-1 (83–89). As found for antibodies to the immune inhibitory checkpoints, and as discussed below, efficacy of immune agonist checkpoint antibodies is regulated by the FcγRs (77–79, 89), with some showing preferential engagement of activatory FcγR (i.e., high A:I ratio), and others of inhibitory FcγR (i.e., low A:I ratio), for optimal therapeutic activity (**Table 1**).

So, what is the common denominator determining FcγR-dependency, and preferential engagement of inhibitory vs. activating FcγR for efficacy of individual targets and antibodies? In a recent landmark paper, Beers and co-workers used a multi-pronged approach to study molecular and cellular FcγR-dependent mechanisms underlying therapeutic activity of antibodies to the co-stimulatory immune checkpoint 4-1BB (89). Firstly, the authors used anti-4-1BB antibodies with identical Fv-regions but differing in isotype–therefore targeting the same epitope on 4-1BB but showing preferential engagement of activating (mouse IgG2a, high A:I ratio) or inhibitory (mouse IgG1, low A:I ratio) antibody checkpoints. Second, effects were studied in immunocompetent tumor-bearing animals differing only by FcγR repertoire–expressing only activating, only inhibitory or both activating and inhibitory antibody checkpoints. Using this approach, the authors found that anti-4-1BB antibodies can stimulate anti-tumor immunity by different mechanisms; Boosting of effector $CD8^+$ T cells, or depletion of regulatory T cells (**Figure 4**). Both mechanisms were regulated by antibody interactions with FcγR, but differently so.

Anti-4-1BB antibodies' depletion of intratumoral Treg cells was shown to be dependent on activating FcγR (89). Antibody isotypes with high A:I ratio showed enhanced Treg deletion, and Treg deletion was diminished in animals lacking activating Fc gamma receptors. A similar dependence on activating antibody checkpoints for Treg depletion had previously been demonstrated for antibodies to other immune receptors e.g., GITR, OX40, CD40, CTLA-4, or IL-2R, i.e., independent of specificity for co-stimulatory or inhibitory immune checkpoints (**Table 1**).

Conversely, boosting of $CD8^+$ T cell responses was most pronounced with antibody isotypes of low A:I ratio. The mechanism underlying enhanced $CD8^+$ T cell responses likely involves FcγRIIB-mediated antibody cross-linking, and thereby promoted signaling, of antibody-targeted co-stimulatory 4-1BB receptors on $CD8^+$ T cells. Agonist anti-tumor activity of anti-CD40 antibodies has previously been proposed to rely on FcγRIIB-mediated antibody cross-linking and promoted signaling in CD40-expressing antigen presenting cells (23, 24) (**Table 1**; **Figure 2B**).

Interestingly, the authors found that concurrent administration of equal doses of high A:I variant (mIgG2a), Treg-depleting, anti-4-1-BB antibodies, and low A:I variant (mIgG1), $CD8^+$ T cell boosting, anti-4-1-BB antibodies reduced therapeutic efficacy. In contrast, sequential administration of first activating FcγR-optimized antibody to deplete Tregs, followed by inhibitory FcγR-optimized antibody to agonize $CD8^+$ T cells, enhanced therapeutic efficacy compared to single agent treatment. These observations indicated competing mechanisms of high A:I antibody mediated Treg depletion, and low A:I antibody mediated $CD8^+$ T cell boosting. This notion that was corroborated through a series of complementary experiments. In short, although the two studied isotype variant antibodies show preferential binding to activatory (mIgG2a, high A:I ratio) and inhibitory (mIgG1, low A:I ratio) FcγRs, respectively, both antibody variants will co-engage activating and inhibitory FcγRs *in vivo*, where their "preferred" type (activating or inhibitory) of FcγR on effector cells is limited in numbers, relative to target cell coated antibody Fc's available for FcγR engagement. Therefore, concurrently administered high A:I ratio and low A:I ratio antibodies will compete for binding to available activating and inhibitory FcγR, resulting in a "frustrated system" of suboptimal Treg depletion and suboptimal $CD8^+$ T cell boosting.

Importantly, if translated to human, these findings could have broad implications for cancer immunotherapy. Human IgG1 and IgG4 antibodies–two of the most common isotypes used in cancer immunotherapy–bind human activating and inhibitory FcγRs with rather similar affinity, compared with the more "polar" affinities of mIgG2a and mIgG1 for activating, and inhibitory FcγRs, respectively. Human IgG1 and IgG4 might therefore be expected to be quite sensitive to such competition, which could help explain the poor translation of promising mouse data to the human clinical setting. Further, the findings are likely relevant to other signaling antibody targets, most notably co-stimulatory receptors of the TNF receptor superfamily. Earlier studies had reported decreased efficacy following concurrent treatment with antibodies to OX40 and PD-1, although underlying molecular mechanisms were not studied (88).

Collectively, these observations shed important light on how antibody checkpoints regulate mechanisms common to cancer cell direct-targeting and immune checkpoint targeting antibodies. Therapeutic activity of either type of antibody may rely principally on target cell depletion (e.g., anti-CD20 or anti-IL-2R), cell depletion and block of target receptor signaling (e.g., anti-Her2 or anti-CTLA-4), or strictly on receptor/ligand blockade e.g., anti-PD-1 (**Figure 5**). Thus, classification of antibodies into cancer cell-direct targeting, immune checkpoint blocking, or immune checkpoint agonists, is inadequate and needs revision (98). Instead, careful dissection of individual antibodies' mechanism(s) of action with respect to their ability to block or agonize receptor signaling and/or deplete target cell(s), and their regulation by interactions with

TABLE 1 | Antibody checkpoints determine efficacy and mechanism-of-action of immune modulatory antibodies.

Antibody MoA		Co-stimulatory checkpoints					Co-inhibitory checkpoints		
		GITR	OX40	4-1BB	CD40	IL-2R	CTLA-4	PD-1	PD-L1
High A:I ratio	Effect	Treg depletion			$CD40^{+}$ cell depletion	Treg depletion	Treg depletion	*FcγRs reduce efficacy	TAM depletion?
	FcγR-modulation	aFcγR↑ iFcγR↓	aFcγR↑	aFcγR↑ iFcγR↓	aFcγR↑ iFcγR↓	aFcγR↑ iFcγR↓	aFcγR↑ iFcγR↓		aFcγR↑ iFcγR↓
Low A:I ratio	Effect	Teff costimulation			APC costimul.				
	FcγR-modulation		aFcγR↓ iFcγR↑	aFcγR↓ iFcγR↑	aFcγR↓ iFcγR↑				
FcγR-indep.	Effect						Block Teff suppression		
mAbs	Isotype(s)	rIgG2b	mIgG1	mIgG2a, mIgG1	mIgG1, hIgG1/2/SE/SELF/V9/V11	rIgG1, mIgG2a	haIgG, hIgG1	mIgG1/2a/$^{1}_{D265A}$, rIgG1, hIgG4	mIgG1/2a/$^{1}_{D265A}$
	Clone(s)	DTA-1	OX86	LOB12.0	1C10, 3/23, FGK45, CP-870,893	PC-61	9H10, 4F10, 9D9, ipilimumab	4H2, RPMI-14, nivolumab, pembro	14D8

*Table indicates antibody Mechanism-of-Action (**MoA**) as a function of antibody isotype preferential engagement of activating (**High A:I ratio**) or inhibitory (**Low A:I ratio**) antibody checkpoints. Mechanisms of immune modulatory antibodies to co-stimulatory immune checkpoints, co-inhibitory immune checkpoints or the IL-2R are indicated. **Effect** indicates main cell type and function identified as underlying therapeutic effects of High A:I, and Low A:I variant antibodies, respectively. **FcγR-modulation:** arrows indicate how activatory FcγR (aFcγR) and inhibitory FcγR (iFcγR) positively (↑) or negatively (↓) regulate indicated effect. Bottom two lines indicate antibody isotypes and clones used in referenced studies. **References**: **GITR** (79), **OX40** (90–95), **4-1BB** (89), **CD40** (23, 24, 96), IL-2R (97), **CTLA-4** (76–80), **PD-1** (81, 82), **PD-L1** (81).*

the antibody checkpoints, will be critical for identification and rational combination of antibodies with complementary non-competing mechanisms-of-action (**Table 1**). As discussed below, such knowledge will additionally pave the way for antibody-checkpoint targeted therapies, e.g., antibody blockade of inhibitory FcγRIIB or Fc-engineering for enhanced affinity to activating FcγR, to help boost efficacy and overcome resistance in the immune suppressed tumor microenvironment.

Targeting the Antibody Checkpoints to Improve Cancer Immunotherapy–Focus on FcγRIIB

The documented role of the antibody checkpoints as master regulators of the clinically most relevant classes of anti-cancer antibodies detailed above, suggests that targeting of this receptor family be an attractive strategy to enhance efficacy and overcome resistance to antibody-based cancer immunotherapy.

While Fc gamma receptor regulation of antibody efficacy is highly functionally conserved between mouse and man, important differences in absolute and relative binding affinities of the species' respective antibody subclasses for their corresponding activating and inhibitory FcγRs have slowed translation into human therapeutic antibody candidates and clinical development. Recent development of FcR-humanized mouse models (99), and highly specific antagonist or agonist antibodies to individual human and mouse activating and inhibitory receptors (30, 65), have now enabled such translation.

Two principal strategies to better harness antibody checkpoint-dependent antitumor immunity have been pursued–Fc engineering or FcγR blockade (**Figure 6**). Antibody engineering to enhance affinity for activating antibody checkpoints has obtained clinical proof-of-concept through the afucosylated CD20-specific antibody obinutuzumab (15), with additional afucosylated antibodies in late stage clinical development (100). While clinically validated, and elegant in the sense that simple removal of a fucose group of residue N297 in the antibody constant domain results in very significantly enhanced binding to FcγRIIIa (101), this approach has its limitations. Firstly, emerging data indicates that intratumoral macrophages and dendritic cells–critical effectors underlying antibody-induced antitumor immunity (102)–express FcγRIIA and FcγRIIB at highest density (76). Further, FcγRIIA may be the only activating Fc gamma receptor expressed on human dendritic cells, which additionally express FcγRIIB for coordinate regulation of antigen presentation (45). Consequently, harnessing the full potential of antibody checkpoint-regulated anti-cancer immunity is likely to require engagement and enhancement of additional activating FcγRs besides FcγRIIIa, and ideally reduced or no engagement of the inhibitory antibody checkpoint. As discussed below, the great structural similarity between individual activating and inhibitory antibody checkpoint receptors poses significant technical challenges to succeed in engineering of antibodies with such properties. Nevertheless, Fc-engineering by substitution of two or more amino acids has generated antibody molecules with enhanced affinity for both FcγRIIA and FcγRIIIA, albeit with retained or slightly enhanced affinity also for the inhibitory FcγRIIB (103, 104). Whether such molecules will show therapeutically relevant pharmacokinetics or enhanced efficacy remains to be demonstrated in clinical trials.

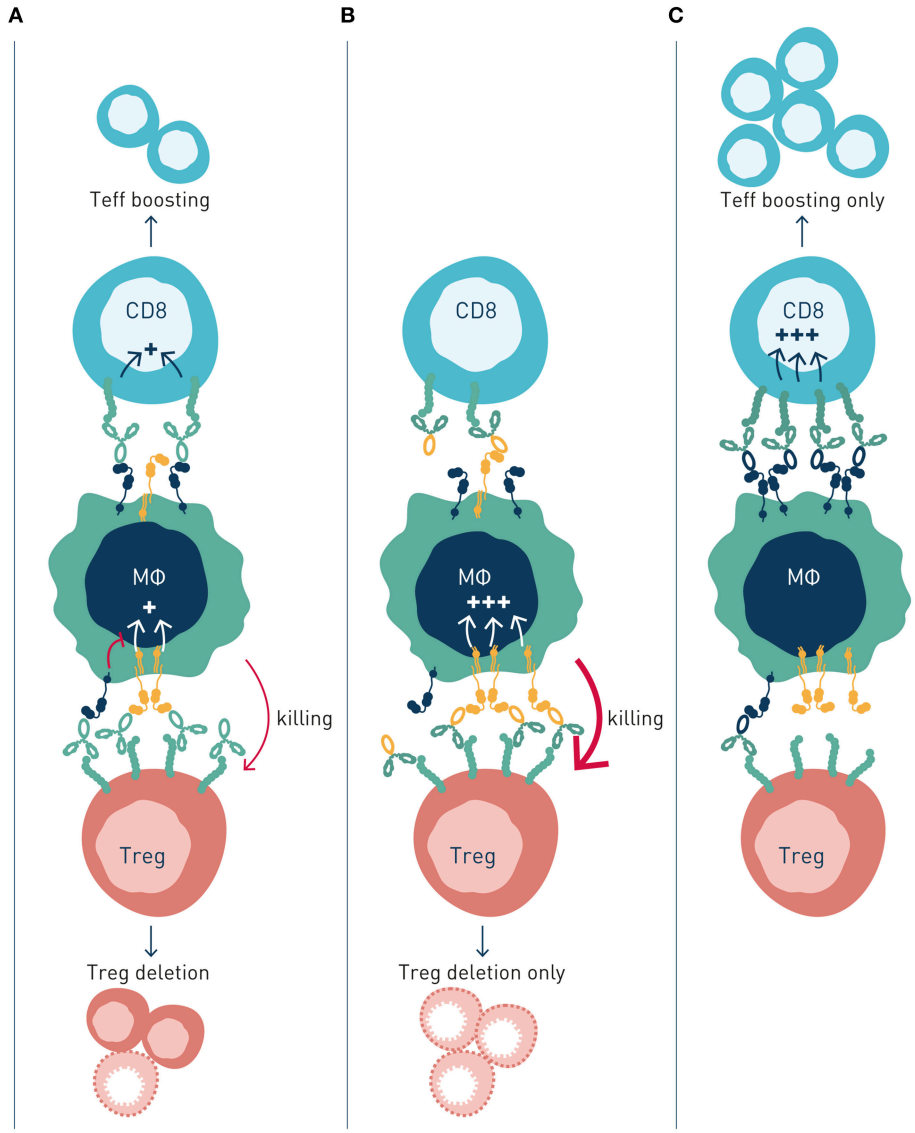
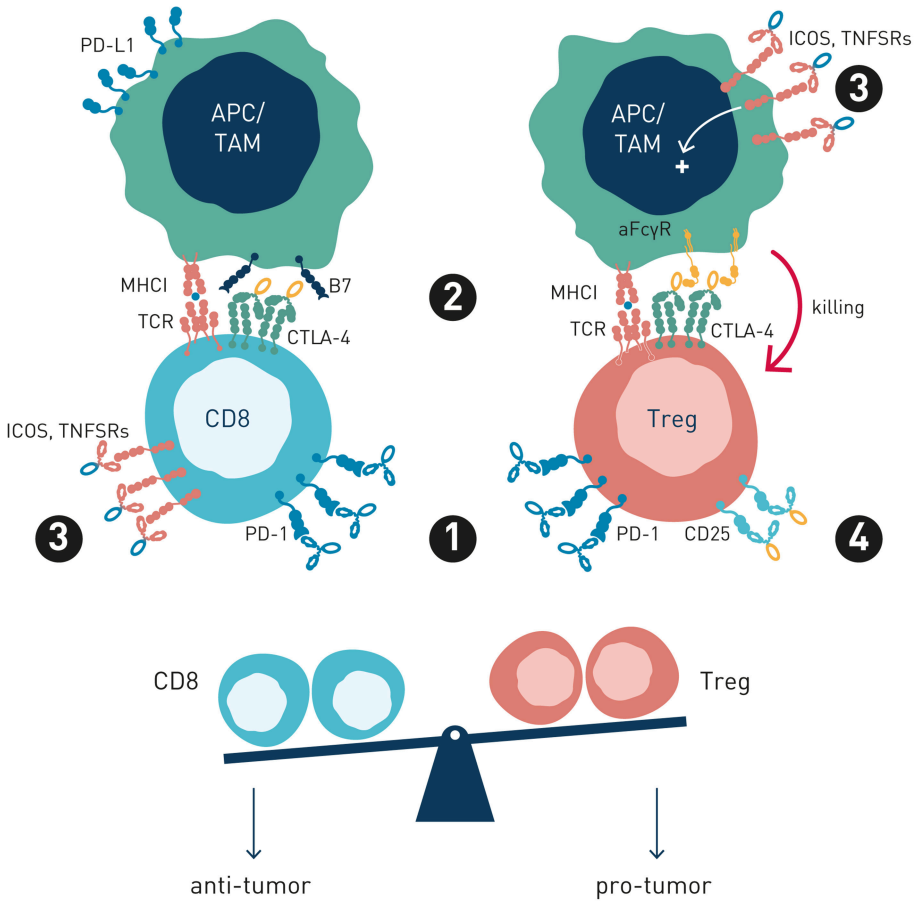


FIGURE 4 | Activating and inhibitory antibody checkpoints determine efficacy and mechanism-of-action of immune agonist antibodies. This schematic figure models **(A)** Antibody engagement of activating and inhibitory FcγRs determine target cell depletion and agonism, respectively. The two mechanism compete when antibody variants (isotype) capable of binding both FcγR are used, resulting in reduced or no therapeutic activity. **(B)** Antibody variants with enhanced binding to activating FcγR (high A:I ratio) show improved depletion of Treg cells, which express higher numbers of receptors compared with effector cells, resulting in immune activation through elimination of suppressor cells. **(C)** Antibody variants with enhanced binding to inhibitory FcγR (low A:I ratio) show improved $CD8^+$ T cell agonism, resulting in immune activation by expansion and boosting of effector cells.

Based on the significant upregulation of the sole inhibitory antibody checkpoint FcγRIIB in the tumor microenvironment (97), and its documented role in conferring resistance to antibody-based therapy in this niche (65, 70, 97), we have pursued antibody-mediated blockade of FcγRIIB as an alternative and complementary approach to Fc-engineering to harness the full potential of antibody checkpoint-regulated immunity. In theory, besides being an apparent critical pan-antibody regulator conferring antibody drug resistance in the tumor microenvironment, targeted blockade of FcγRIIB by a separate antibody has the advantage of enabling combination therapy and boosted efficacy with multiple existing, clinically validated, antibodies including those engineered for enhanced binding to activating FcγR (65). The strategy does, however, put exquisite requirements on a therapeutic antibody candidate, both from target receptor specificity and function-modulating perspectives. The extracellular, antibody accessible domain, of the inhibitory FcγRIIB is ∼93% homologous with the activating FcγRIIA. Nevertheless, probing of a highly diversified human recombinant antibody library (65), or immunization of mice transgenic for human FcγRIIA (105), generated diverse pools of highly specific



MoA	block ligand receptor	aFcγR engaging	iFcγR engaging	
1. blocking mAb	+	−	−	
2. block & deplete	+	+	−	
3. agonist	−	−	+	
4. deplete only	−	+	−	

FIGURE 5 | Antibody preferred mechanism-of-action and FcγR-engagement is dependent on target receptor function and expression. This schematic figure models four exemplary antibody MoA's, pertinent to both immune checkpoint and tumor cell direct-targeting antibody types. (1) Blocking mAb. PD-1, a co-inhibitory antibody checkpoint expressed at high and similar levels on intratumoral Treg and Teff cells, is best targeted using a PD-1/PD-L1 blocking Fc-null antibody variant, since FcγR-mediated Teff cell depletion is undesirable (2) Blocking and depleting mAbs. Anti-CTLA-4 is overexpressed on intratumoral Treg compared with Teff, and activatory FcγR-engagement correlates with survival in melanoma patients treated with ipilimumab. Preferred MoA is two-fold: CTLA-4/B7 blockade and Treg depletion through FcγR-dependent mechanisms (3) Agonist mAb Preferred MoA is FcγR-engaging antibody variant, where FcγRs promote receptor cross-linking and signaling. (4) Depletion only mAb. Anti-IL2R antibody preferred MoA is ligand non-blocking and FcγR-dependent (Treg) cell depletion. IL-2R overexpressing Tregs are selectively depleted, while free IL-2 may promote Teff survival and expansion.

antibodies that selectively bound to FcγRIIB, and not to FcγRIIA, and which in a dose-dependent manner blocked immune complex binding to cell surface-expressed FcγRIIB. Functional screening revealed that only a minority of the highly FcγRIIB specific human recombinant antibodies were able to block antibody-induced FcγRIIB inhibitory signaling (65). Remaining candidates either did not block, or agonized, FcγRIIB signaling. The latter category could have therapeutic potential in treatment of chronic inflammatory and autoimmune disease (106).

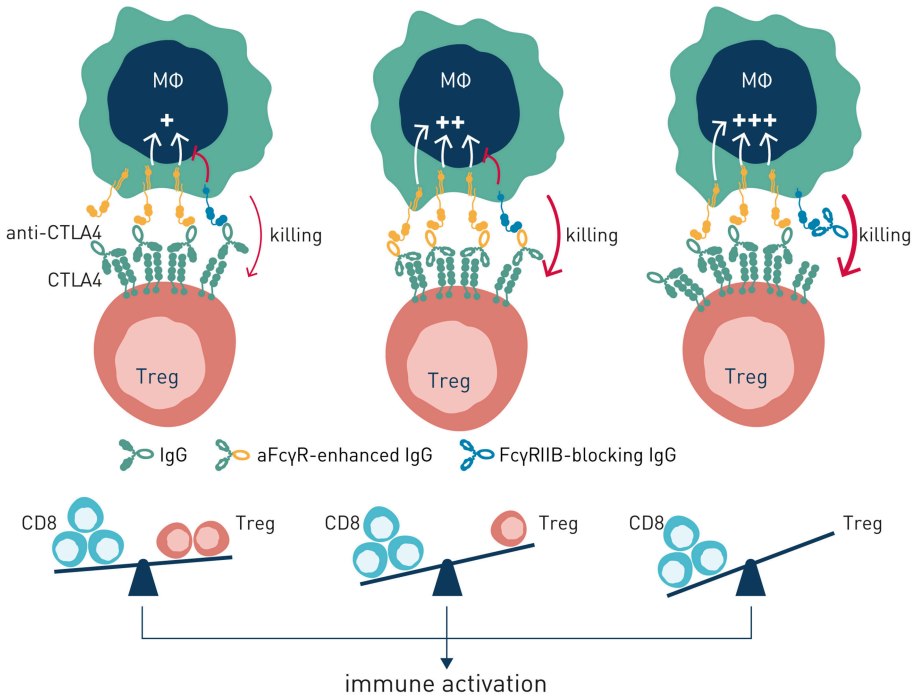


FIGURE 6 | Antibody-induced antitumor immunity can be enhanced by modulation of antibody: FcγR interactions. **Left** panel (no antibody checkpoint modulation). Antibody efficacy is balanced by co-engagement of activating and inhibitory FcγR. **Center** panel (enhanced engagement of activating FcγR). Antibody efficacy is improved through Fc-engineering for enhanced binding to activating FcγR. **Right** panel–Antibody efficacy is enhanced by blockade of the inhibitory FcγRIIB.

Based on observations that FcγRIIB limits antibody efficacy and promotes tumor cell resistance by dual mechanisms in B cell malignancy, acting at the level of both immune effector cells and tumor B cells, we have further characterized the therapeutic potential of antagonistic anti-FcγRIIB antibodies to boost efficacy and overcome resistance to antibody therapy *in vivo* focusing initially on this setting. A lead human antagonistic anti-FcγRIIB IgG1 antibody (6G11 or BI-1206), which showed synergistically enhanced rituximab B cell depletion in FcγRIIB and CD20 humanized mice, and overcame refractoriness of primary leukaemic B cells to anti-CD20-based antibody therapy *in vivo*, is currently in early phase clinical testing (65).

Besides affording efficacy, therapeutic targeting of Fc gamma receptors, whether by blocking antibodies or Fc-engineering, must be safe and associated with therapeutically relevant pharmacokinetics. In addition to its high expression on B cells and certain macrophage/dendritic cells, FcγRIIB has been reported to be highly expressed in mouse and rat liver sinusoidal endothelial cells (LSEC) (107), where they have been implicated in removal of circulating small immune complexes (108). These observations raise potential safety concerns of undesirably cytotoxic activity with therapeutic antibodies targeting FcγRIIB. However, our recent observations of human and mouse liver indicate lower LSEC expression in man (30), and dosing of FcγRIIB humanized mice with therapeutically relevant doses of anti-human FcγRIIB IgG1 antibody 6G11 showed no apparent acute or chronic treatment related adverse effects (30, 65). Ultimately, the safety and efficacy of targeting FcγRIIB needs to be assessed in human subjects. Two clinical trials are ongoing to evaluate safety and explore efficacy of the BI-1206 antibody as single agent and in combination with rituximab in B cell malignancy (NCT03571568 and NCT02933320). Our ongoing efforts aim at translating observations of FcγRIIB-regulated antitumor immunity to the solid cancer clinical setting.

As noted above FcγRIIB may promote anti-tumor activity by facilitating extrinsic signaling of certain co-stimulatory receptors expressed on tumor or immune cells. A possible strategy to enhance therapeutic activity of such antibodies would therefore be to enhance their affinity for FcγRIIB. In keeping with this, anti-DR5 antibodies carrying the S267E ("SE") mutation, increasing human IgG1 affinity for FcγRIIB several hundred-fold, showed improved tumor regression in mouse models humanized for FcγRIIB (109). Analogously, human IgG2 anti-CD40 antibodies equipped with SE or SE/LF mutated backbones (the latter further increases affinity for FcγRIIB) showed enhanced $CD8^+$ T cell activation, and improved ability to clear tumors, in mice humanized for FcγRs and CD40 (96). However, increasing antibody affinity for FcγRIIB in these two cases improved not only efficacy but also side effects. Increased DR5 agonism of the SE variant anti-DR5 was associated with increased liver enzyme release. SE and SE/LF variant anti-CD40 antibodies increased not only T cell activation and anti-tumor immunity, but also

depletion of platelets, which express CD40 (96, 109). Thus, Fc-engineering for enhanced FcγRIIB affinity or selectivity needs close consideration of antibody (Fv-) targeted receptor's cellular distribution and function(s).

CONCLUDING REMARKS

Emerging preclinical and clinical data demonstrate that the activating and inhibitory Fc gamma receptors–the "antibody checkpoints"–control antitumor immunity induced by the clinically most successful antibodies used in cancer immunotherapy. Therapeutics that harness the power of antibody checkpoint-regulated anti-tumor immunity, through Fc-engineering to enhance binding to activating FcγRs, or through blockade of the inhibitory FcγRIIB, have been approved or are in development. If safe and well-tolerated, these agents hold promise to improve response rates, duration of response, and potentially overall survival for diverse cancer patients.

AUTHOR CONTRIBUTIONS

BF wrote and edited the manuscript and conceived figures. IT helped write the manuscript and conceive figures. LM designed and performed experiments in several of herein reviewed papers, and helped write the current manuscript and helped conceive figures.

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REVIEW
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Extra-Neutralizing FcR-Mediated Antibody Functions for a Universal Influenza Vaccine

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While neutralizing antibody titers measured by hemagglutination inhibition have been proposed as a correlate of protection following influenza vaccination, neutralization alone is a modest predictor of protection against seasonal influenza. Instead, emerging data point to a critical role for additional extra-neutralizing functions of antibodies in protection from infection. Specifically, beyond binding and neutralization, antibodies mediate a variety of additional immune functions via their ability to recruit and deploy innate immune effector function. Along these lines, antibody-dependent cellular cytotoxicity, antibody-mediated macrophage phagocytosis and activation, antibody-driven neutrophil activation, antibody-dependent complement deposition, and non-classical Fc-receptor antibody trafficking have all been implicated in protection from influenza infection. However, the precise mechanism(s) by which the immune system actively tunes antibody functionality to drive protective immunity has been poorly characterized. Here we review the data related to Fc-effector functional protection from influenza and discuss prospects to leverage this humoral immune activity for the development of a universal influenza vaccine.

Keywords: influenza, antibody, Fc receptor, vaccine, ADCC, glycosylation, adjuvant

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INTRODUCTION

Influenza viruses are enveloped negative-strand RNA viruses with segmented genomes that can infect a variety of birds and mammals, including humans (1). Seasonal influenza affects 10–20% of the world's population per year (2), which is estimated to cost $4.6 billion yearly for hospitalizations, doctor's visits, and medications in the United States alone (3). Additionally, influenza causes U.S. employees to miss approximately 17 million workdays due to flu, at an estimated cost of $7 billion a year in sick days and lost productivity (3). Increased infection and mortality occurred during four pandemics in the 20th and 21st centuries, in 1918, 1957, 1968, and 2009 (4), and could occur again if a new strain, such as avian influenzas H5N1 or H7N9, begins to circulate in the human population.

To address this looming threat, the National Institute of Allergy and Infectious Diseases (NIAID) has named the development of a universal influenza vaccine, defined as one that provides protection against symptomatic disease from ≥75% of influenza A strains, as one of its research priorities (5). A key component of the strategic plan is the initial characterization of the correlates of immunity against influenza infection and disease (6). While neutralization has been widely considered the major protective correlate of immunity, hemagglutinination-inhibiting neutralizing antibodies alone have been only modestly linked to protection from seasonal influenza infection,

suggesting the involvement of extra-neutralizing antibody functions (7–10). Moreover, currently licensed seasonal influenza vaccines provide only moderate (10–60%) protection against specific strains of seasonal influenza, and little to no protection from emerging pandemic influenza (11). This low efficacy is caused by subtype and strain variability of the two major viral antigens, hemagglutinin (HA) and neuraminidase (NA), as well as by antigenic drift (12). All the available seasonal influenza vaccines, including the inactivated influenza vaccine (IIV), adjuvanted IIV (FluAd), and live attenuated influenza vaccine (LIAV), are given yearly due to limited response durability and the need to induce *de novo* immunity to novel circulating strains. The development of yearly influenza vaccines relies on predictions published by the World Health Organization to determine the strain composition for a given year (13). Due to the long lead times in producing adequate quantities of the vaccine, the strains must be selected roughly 6 months in advance of vaccine administration (13), leading to population vulnerability should a new strain enter circulation. When these predictions did not match seasonal circulating strains, the effectiveness was very low (11).

Beyond efforts to match sequences to ensure seasonal immunity, the current surrogate of protection used to evaluate influenza vaccines is the hemagglutination inhibition (HAI) assay (14). HAI was identified as a predictor of protection from infection in the initial study of egg-grown inactivated vaccine efficacy conducted in 1943 by Salk et al. (15). HAI measures the ability of an antibody or serum sample to prevent HA binding to red blood cells, and is considered a proxy for neutralization by receptor blockade (14). HAI, however, does not fully explain or predict protection in humans (7–9). Indeed, individuals lacking detectable HAI titers were found to be resistant to influenza infection. Additionally, infection risk was clearly linked to age independently of HAI titer (7). While HAI is considered a classical surrogate of protection from influenza infection, it alone is not sufficient to fully explain protection (10).

Humoral immune responses to influenza are largely directed toward the hemagglutinin molecule (HA). HA, the primary viral glycoprotein, exists as a trimer made of monomers composed of two subunits, HA1, roughly corresponding to the "head" and HA2, or "stem" (16) (**Figure 1**). Heterosubtypic or cross-reactive, antibodies to the HA head region are relatively rare due to heavy glycosylation and low sequence conservation in this region (17, 18). Although highly variable, cross-reactive neutralizing antibodies have been discovered against the HA head. However, these antibodies bind primarily to specific conserved epitopes, including the receptor binding domain (18). In contrast, while several protective antibodies have been identified against the HA stem, as it is more conserved, this region of the HA is poorly immunogenic (17).

Heterosubtypic protective antibodies against influenza primarily target either the receptor binding site on the HA head or the more conserved HA stem (19). However, the stem region is infrequently targeted compared to easily inducible strain-specific HA head responses (20). Regardless of target, antibodies against HA can mediate protection by neutralization or extra-neutralizing functions, and both modalities may be exploited by a single antibody specificity. Antibodies that target the head largely provide protection by preventing the virus from entering the target cell, and are thus referred to as neutralizing antibodies (19). Non-neutralizing functional protective influenza-specific antibodies have been documented against both the HA head and stem (21, 22); however, their mechanisms of action are more complex and varied (23). Although protective non-neutralizing antibodies have been documented across the HA molecule, these types of protective antibodies more dominantly target the stem region of HA and can exhibit wide reactivity, capturing most influenza A viruses (20). Because the neutralizing capacity of antibodies is dose-dependent (21), in this review the term non-neutralizing will be used to describe antibodies that cannot efficiently neutralize virus at the concentration currently being studied.

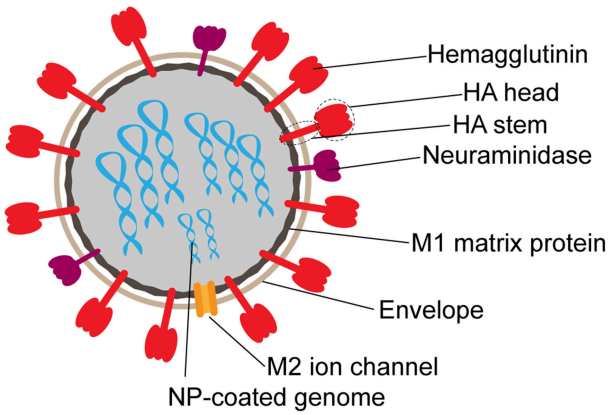

FIGURE 1 | Schematic structure of influenza virion. Surface proteins hemagglutinin (HA) and neuraminidase (NA) are present on the surface at an approximate 3:1 ratio. The M2 ion channel also spans the envelope. M1 matrix protein forms the inner capsid, which surrounds the segmented RNA genome coated in nucleoprotein (NP).

While the mechanism of protection mediated by neutralizing antibodies is simple to comprehend, the extra-neutralizing mechanisms of action of antibodies are less well-understood. Emerging evidence has suggested that mechanisms including the ability of antibodies to leverage the innate immune system may contribute to protection against influenza (21–31). Critically, antibodies possess two functional domains: the Fab, which recognizes the antigenic epitope, and the Fc, which interacts with Fc receptors (FcR) or complement to drive antibody-mediated effector functions (**Figure 2**). Passive transfer studies using both native IgG1 and FcR-binding ablated monoclonal antibodies (mAbs) clearly illustrated the importance of Fc-mediated functions in protection from infection (21, 22). Moreover, follow-up studies using FcR and complement knockout mice further clarified the critical nature of specific Fc-effector functions in protection (21–23, 25–31). Antibody mediated macrophage phagocytosis (28, 32), neutrophil production of reactive oxygen species (28), cellular cytotoxicity (29), and complement deposition (26, 27, 32, 33) have all been implicated as protective functions leveraged by antibodies to drive

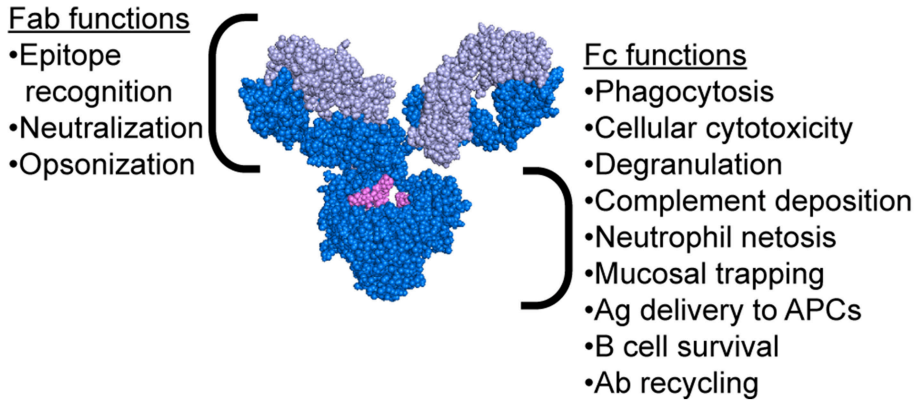

FIGURE 2 | Antibody structure highlighting functions of both the Fab and Fc regions. Antibody image shows heavy chain in dark blue, light chain in light blue, and glycan in magenta. Antibody structure: PDB 1IGY.

protection from infection and/or viral clearance. Strikingly, even broadly neutralizing HA-stem targeting and pan-strain HA-head targeting mAbs require FcRs to confer protection (22). In this review, we explore the various mechanisms beyond neutralization that are exploited by antibodies to confer protection from influenza and promote viral clearance.

FCR-MEDIATED FUNCTIONS IN INFLUENZA INFECTION AND VACCINATION

Antibody-Dependent Cellular Cytotoxicity (ADCC) by Natural Killer (NK) Cells

Antibody-dependent cellular cytotoxicity (ADCC) is largely mediated by the interaction of pathogen or cell-surface bound antibodies with Fc gamma receptor IIIa (FcγRIIIa) on NK cells in humans (**Figure 3D**) and FcγRIV on monocytes, macrophages, and neutrophils in mice (34, 35). FcγRIIIa is found on the surface of human NK cells, monocytes, and macrophages (36). Engagement of FcγRIIIa by antibody causes the release of cytotoxic granules from NK cells, uptake by macrophages, apoptosis of infected targets, and secretion of antiviral cytokines and chemokines (37–40).

It was first reported in 1977 that human peripheral blood leukocytes engaged in influenza-specific ADCC, and that this effect correlated with antibody-mediated virus neutralization (41, 42). *In vitro* ADCC activity was linked to protection conferred by antibodies in a mouse model of influenza infection (21, 43). Mice that received an FcR-binding competent antibody capable of inducing ADCC *in vitro* (measured by CD107a expression) exhibited increased survival and decreased morbidity compared to mice that received either an antibody unable to bind to FcRs or a less potent ADCC-inducing antibody (21). Similarly, macaque models of repeated influenza infection confirmed the rapid development of ADCC following infection in animals previously exposed to influenza. This highlighted the presence of antibodies in the bronchoalveolar lavage (BAL) capable of inducing activation of NK cells, which were associated with increased viral clearance and decreased duration of disease (44). Analogously, in human studies, ADCC titers were associated with a reduction in disease burden in a seasonal influenza experimental infection study (45). Additionally, older adults, who had previously seen 2009 pandemic influenza-like viruses in the past, and who retained long-lived ADCC, but not neutralizing, antibody titers, were protected during the 2009 H1N1 pandemic (46). This provided further evidence that ADCC-mediating antibodies are associated with protection.

However, despite our emerging appreciation for the potential role of ADCC in protection from infection and disease, the seasonal influenza vaccine poorly induced broadly reactive ADCC-inducing antibodies in healthy children and adults (45, 47–49). Conversely, the presence of cross-reactive HA-specific antibodies that can activate NK cells in older adults suggests that these functional antibodies accumulate over the course of many years of repeated natural infection with influenza (48, 50). Despite the delay in their evolution, the data clearly suggest that these functional cross-reactive antibodies emerged naturally over time. Moreover, some healthy American adults possessed ADCC activity against avian H7N9 and H5N1 viruses that do not circulate in North America but could cause pandemic outbreaks. This indicated the natural evolution of cross-reactive functional antibodies targeting diverse HA antigens in the absence of exposure and/or other conserved viral proteins such as NP and M2 (31, 51). Furthermore, broadly cross-reactive ADCC-inducing antibodies were reported in individuals who lack broadly neutralizing influenza-specific antibodies (46, 50–52), suggesting that these functions emerge separately and may evolve under distinct stimuli. Collectively, the data clearly demonstrate that broadly protective ADCC inducing antibodies are associated with protection and evolve naturally over time.

HA head-specific mAbs induced less ADCC than stalk-specific antibodies in an *in vitro* NK cell activation assay (21). This difference in function has been suggested to be related to the inability of the head-specific mAbs to efficiently multimerize when bound to antigen on the cell surface and interact with low-affinity FcRs to induce functional responses (21). A recent study suggested an alternative explanation in experiments using

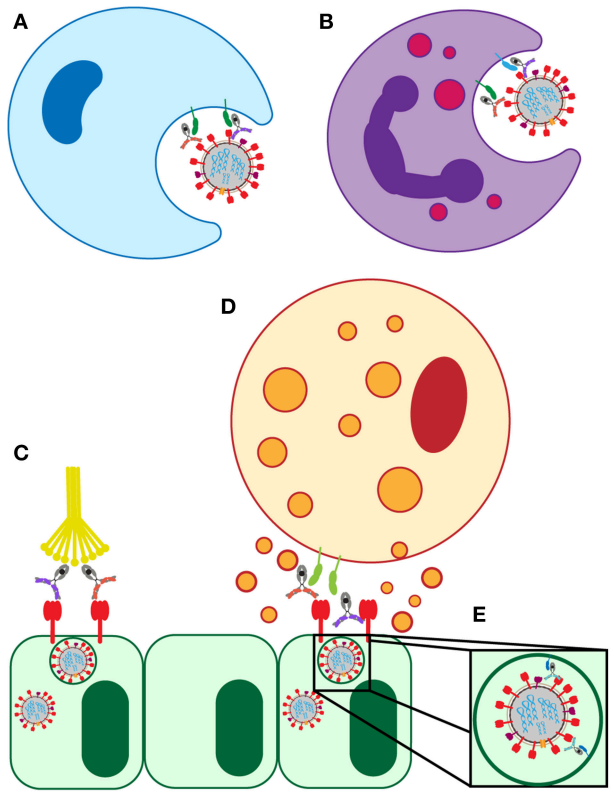

FIGURE 3 | Known FcR-dependent innate immune effector functions acting in influenza infection. **(A)** Clearance of virions and infected cells by macrophage phagocytosis. **(B)** Clearance of virions and infected cells by neutrophil phagocytosis, and the release of cytokines and reactive oxygen species. **(C)** Clearance of infected lung epithelial cells and activation of the adaptive immune system by antibody interaction with C1Q. **(D)** Clearance of infected lung epithelial cells by ADCC. **(E)** Neutralization of virus by FcRn-bound HA-specific antibodies.

FLAG-tagged HA to direct FLAG-specific antibody to certain regions of the HA molecule. The data from this study suggested that two points of contact were required between infected and effector cells for efficient ADCC activity (53). These direct contacts are (1) between the mAb Fc and FcR and (2) between the cell surface sialic acid and viral HA (53). However, the co-ligation of FcR and viral protein has not been borne out by studies in other infection or disease contexts, or in polyclonal pools of antibodies directed against native HA.

An additional layer of complexity in dissecting non-neutralizing antibody mediated mechanisms of protection *in vivo* is the comparison of polyclonal vs. monoclonal mediated antibody functions. Emerging data suggest that the level of *in vitro* ADCC is influenced by the ratio of ADCC-inducing to ADCC-inhibiting antibodies (22, 54). ADCC-inhibiting antibodies, which can be neutralizing, were shown to compete for binding sites on HA on the surface of viral particles and infected cells (22, 54). While the delivery of single protective ADCC-inducing mAbs demonstrated striking protection from infection *in vivo* (21, 22), polyclonal pools of antibodies exhibited a much more complex balance of epitopes targeted and functional competition that collectively may contribute to differential protection from infection during seasonal exposure. While it is clear that functional antibodies play a vital role in protection against influenza infection, experimental approaches able to comprehensively dissect the nature of polyclonal antibody interactions are urgently needed to further define the nature of protective antibody activity and guide vaccine design.

Antibody-Dependent Macrophage Phagocytosis and Activation

ADCC-inducing antibodies, as well as the direct cytopathic effects of the virus, drive infected cell apoptosis (55). These infected apoptosing cells are then cleared through phagocytosis to maintain tissue homeostasis (56). Post-infection, macrophages are rapidly recruited to the lung and are present in BAL, airway, and alveoli to support the rapid clearance of infected and/or dying cells (57). While the supernatant of influenza-infected cells can stimulate monocyte phagocytosis independently of antibody involvement (57), antibodies contribute to accelerated clearance of viral particles and infected cells through interactions with FcγRIa and FcγRIIa on immune cells (58). Antibody mediated viral phagocytosis, resulting in viral degradation, was linked to decreased spread and severity of infection (58). While this mechanism was not directly associated with prevention of infection, it was linked to reduced severity of symptoms and viral shedding, and thus attenuating disease in humans.

Antibody-dependent cellular phagocytosis (ADCP) activity (**Figure 3A**) in healthy human serum, mediated by monocytes/macrophages, was shown to correlate with HAI titer both for circulating and non-circulating strains of influenza (58). Interestingly, ADCP activity was still detectable in diluted serum samples, even at dilutions where neutralization was no long detectable (58). This indicated that phagocytic antibodies may mediate viral clearance even at very low levels, and thus could still provide protection or lessen the severity of disease. Along these lines, non-neutralizing protective mAbs in mice required alveolar macrophages to provide protection. This protection was partially dependent on the induction of a robust inflammatory response in the lung as shown by tissue histology and increased cytokine/chemokine production, and was partially through direct phagocytosis (30). Additionally, broadly neutralizing HA-specific mAbs also exhibited enhanced protection in the presence of alveolar macrophages (30). This macrophage-mediated protection was dependent on interactions of the antibody with FcRs on the macrophage surface, as evidenced by experiments using FcR-binding null antibodies that failed to provide protection from infection (30). Together, these studies indicate that FcR-mediated macrophage activation reduces disease burden and protects mice from lethal influenza, and that healthy human serum has influenza-specific antibodies capable of inducing this function.

Antibody-Dependent Neutrophil Phagocytosis and Activation

Neutrophils are among the first cell populations recruited to the site of infection and/or inflammation, and have been implicated

in the protective response to influenza (59). Neutrophils are involved in the phagocytic clearance of both virions and infected cells, release immunostimulatory cytokines and chemokines to recruit additional immune cells, and form neutrophil extracellular traps (NETs) to capture and inactivate the virus (60). During influenza infection, neutrophils generate the chemokine CXCL12, required for efficient recruitment of cytotoxic $CD8^+$ T cells to the lung (61). However, beyond this indirect antiviral role, human neutrophils express high levels of FcγRIa/b/c, FcγRIIa, and FcγRIIIb after activation, enabling them to respond rapidly and efficiently to antibody coated targets (36). In addition, neutrophils constitutively express the FcαRI, an activating receptor that binds IgA and activates cytotoxic and phagocytic responses via a shared FcR γ-chain (62).

Like macrophages, neutrophils are intimately involved in the phagocytic clearance of infected and apoptotic cells in the lung during influenza infection (**Figure 3B**) (57). Impaired neutrophil phagocytosis through depletion of neutrophils was linked to decreased survival in a mouse model of influenza infection (57, 63). Following phagocytosis, neutrophils form phagolysosomes containing reactive oxygen species (ROS) to eliminate the virus (60). Both HA head- and stalk-specific mAbs induced the production of ROS by neutrophils *in vitro* in an FcR dependent manner, shown following Fc-blockade resulting in reduced ROS production (28). Influenza-specific class-switched IgA antibodies were also implicated in neutrophil activation and ROS production (28). Despite the ability of some antibodies to recruit neutrophil activity *in vitro*, the critical nature of neutrophils in protection from infection remains controversial. Some animal studies using neutrophil depletions finding no significant roles for these cells in protection mediated by passively transferred non-neutralizing mAbs (30), arguing that alveolar macrophages may play a more dominant role in protection. In other studies, neutrophil recruitment and function was linked to protection from infection and reduction in disease (63, 64). Thus, additional studies will be required to ultimately define the role of neutrophils in influenza infection.

Antibody-Dependent Complement Activation

The complement system can recognize and eliminate viruses directly or can contribute to viral clearance via antibody mediated activation (**Figure 3C**) (33). The requirement for complement in protection from lethal influenza infection in mice was established in 1978 and has been more recently replicated on novel influenza strains (26, 65). Influenza virions were shown to be susceptible to both classical and alternative complement mediated lysis *in vitro* only when opsonized by antibodies (27). However, the level of susceptibility varied by strain. Further supporting the involvement of antibody-mediated complement elimination in the influenza immune response, synergy between the classical and alternative complement pathways was shown to provide protection against pandemic H1N1 strains in mice and the cooperativity of both pathways is associated with enhanced viral clearance (27). In these experiments, C3 knockout mice (deficient in all complement pathways because C3 is the central point of the cascade), C4 knockout mice (deficient in classical and lectin pathways), and complement factor B (FB) knockout mice (deficient in alternative pathway) were infected with influenza and disease progression was compared. While both C4- and FB-deficient mice showed increased mortality, neither pathway alone nor the additive mortality approached the level of mortality in C3 knockout mice, who have both pathways of complement ablated. This indicated that the two complement pathways work synergistically to clear infection (27).

Beyond antibody driven virion elimination, the complement protein C3 was also shown to promote higher titers of influenza-specific IgG antibodies. C3 also improved CD4+ and CD8+ T cell responses in the mouse models of influenza infection (33). Vaccination was administered to C3 knockout mice, resulting in dampened antibody titers leading to increased mortality when compared to wild type mice. The role of complement in driving immunity was proposed to be effectuated by the formation of pro-inflammatory complement degradation products C5a and C3a, which can serve a dual role of directly recruiting T cells and enhancing T cell priming by recruiting and stimulating antigen-presenting cells to the site of infection (33).

In human serum, neutralization and complement-dependent lysis activities by mAbs have not always correlated, although neutralizing antibodies can induce complement-dependent lysis (66). Both IgG1 and IgM antibodies have been implicated in the activation of the complement system in influenza infection (27). Complement-stimulating antibodies correlated with protection from infection in children in a serosurveillance study of seasonal influenza (50), which was potentially attributable to their generally higher cross-reactivity compared to neutralizing antibodies (31). Importantly, if complement-inducing antibodies do in fact generally possess higher cross-reactivity when compared to neutralizing antibodies, complement lysis of virus is an attractive strategy for limiting initial infection with influenza by otherwise non-protective non-neutralizing antibodies, broadening the epitopes that can be targeted by vaccination.

Additional Functions Via Non-classical FcR

The neonatal Fc receptor, FcRn, is involved in transcytosing IgG across the placenta during fetal development, across the vascular endothelium to increase extravascular antibody levels, and across the mucosal epithelium to provide humoral defense within the mucosa (67). Additionally, FcRn has a non-canonical role in antiviral immunity against influenza. FcRn was implicated in facilitating antibody-mediated neutralization of influenza virions by HA head-specific antibodies that bind to the virus at acid pH (**Figure 3E**) (68). These unusual head-specific antibodies were then shown to neutralize the virus by preventing trafficking of the viral ribonucleoproteins into the nucleus for replication (68).

Systems level analyses aimed at defining biomarkers of productive immunity to flu vaccination identified pre-existing antibody titers as a negative predictor of response to vaccination (69), thought to act by capturing, destroying, and preventing response to vaccine antigens (historically called original antigenic sin) (70). However, recent studies suggested that pre-existing antibodies shape the immune response to influenza vaccination

in ways that could be utilized to improve protection. Individuals with the most influenza-specific antibody affinity maturation had significant changes in antibody glycosylation, namely increased sialic acid (71). A mechanism was proposed in which pre-existing cross-reactive influenza antibodies, which opsonize incoming vaccine antigen, drove the delivery of immune complexes to germinal centers of lymph nodes by subcapsular sinus macrophages or non-cognate B cells, preferentially when antibodies were sialylated (72, 73). This delivery relied on interaction of immune complexes with the non-canonical IgG Fc-receptor CD23 to capture antigen and move it to germinal center (71). This delivery of antigen to lymph nodes was speculated to increase and extend the contact between B cells and antigens of interest to drive affinity maturation, which can increase both the affinity and potential evolution of neutralization (74, 75). Identified broadly cross-reactive neutralizing mAbs specific to influenza HA were shown to be highly affinity matured, indicating that this pathway may be essential for the development of broad humoral immunity (76).

In addition to trapping and delivery, these sialylated Fcs were also shown to increase B cell inhibitory FcR, FcγRIIb, expression, resulting in elevated thresholds required to activate B cells during development in the germinal center (77). With these elevated activation thresholds, B cells that require higher affinity interactions or an ability to capture more antigen to become fully activated within the germinal center may then experience more aggressive somatic hypermutation and consequent affinity maturation. Sialylated immune complexes bind to non-cognate B cells at high levels in the presence of CD23 (77) to increase affinity maturation. This may offer a novel approach for the design of next generation vaccines able to leverage the potent immunomodulatory activity of the Fc-domain of antibodies.

Together, these data suggest that the quality of the antibody response may not only influence direct antiviral activity, but may also be critical in influencing the response to vaccination. Seasonal influenza infection in humans has been shown to induce moderate antibody cross-reactivity (78), and these pre-existing cross-reactive antibodies may be molding the affinity maturation of new antibodies following vaccination through mechanisms that increase somatic hypermutation, including increased antigen retention within germinal centers (71). Harnessing, increasing, and improving this pathway presents a novel method of improving the breadth and binding affinity of antibodies following influenza vaccination.

CONTROL OF FCR-MEDIATED FUNCTIONS BY ANTIBODY PROPERTIES

Subclass and Isotype Variation

The functional potency of an antibody is significantly affected by the antibody's subclass, which determines the binding affinity of the antibody for FcRs (79). Antibody function is determined at the time of B cell programming via class switch recombination (IgA, IgM, IgG, IgD, or IgE; **Figure 4**). Because each isotype can interact with a distinct family of FcRs present on innate immune cells within disparate compartments, each isotype has the capacity to drive unique antibody effector functions. Beyond the isotypes, there is additional capacity to select for subclasses of particular antibody isotypes. In humans, four IgG subclasses can be additionally selected during an immune response, each of which have further differential affinities for individual FcRs (36). IgG1 antibodies are the most prevalent at approximately 65% of total serum IgG, with the other three subclasses in decreasing fractions in numerical order (80). Because individual subclasses have different affinities for FcRs (36), subclasses drive different antibody effector functions. IgG1 and IgG3 are considered to be the most functional subclasses due to their enhanced ability to bind to FcRs, while IgG2 and IgG4 have lower affinities for FcRs (81, 82). However, IgG3 has a shorter half-life, related to decreased binding affinity to FcRn and due to a proteolytically vulnerable hinge, although multiple allotypes of IgG3 with longer half-lives have been reported among non-Caucasian populations (83).

The relative magnitude and distribution of IgG subclass responses vary between acute influenza infection and vaccination. Vaccination increased IgG3 production when compared to acute infection in adults and children who were previously exposed to natural influenza but not previously vaccinated (84). IgG3 levels following seasonal influenza vaccination correlated with cytokine production by peripheral blood mononuclear cells (PBMCs) stimulated *ex vivo* with infectious influenza virus, suggesting that enhanced IgG3 responses were a marker of a more effective response to vaccination (80). While IgG3 is widely considered to be the most functional subclass due to its affinity for FcRs, the specific effects of IgG3 in protection from influenza remain largely unclear.

While IgG is present at higher levels in the blood, IgA antibodies are produced at considerable levels in mucosal tissues (85). Secreted IgA represents ∼70% of the body's total Ig production and, in mucosa, is primarily dimeric, with only small fractions of monomer, trimer, and tetrameric IgA in the mucosa. In serum, IgA is primarily monomeric (86). Mucosal IgA can prevent influenza infection in the nasal and upper respiratory mucosa with higher heterologous neutralization than IgG with the same Fab (85, 87–89). The protective activity of IgAs was linked to both direct viral neutralization as well as viral capture and cross-linking to the mucosal surface, preventing cell entry in the absence of classical neutralization (85). However, beyond its direct antiviral effects, IgA may also recruit the indirect activity of the innate immune system, via the Fc-receptor for IgA, FcαRI, which is constitutively expressed on neutrophils and increases in expression as neutrophils mature (62). Stimulation of this receptor by influenza-specific IgA was linked to increased ROS production (28), although the precise effect of this activation during influenza infection is unclear.

Antibody Fc Glycosylation

Beyond isotype and subclass selection, the humoral immune response additionally modifies antibodies via post-translational changes in Fc-glycosylation to further tune antibody affinity for FcRs, and thus to modulate antibody effector function (90). Each IgG molecule contains two N-glycosylation sites, at asparagine 297 (N297) on each heavy chain (**Figure 4**). The core Fc glycan

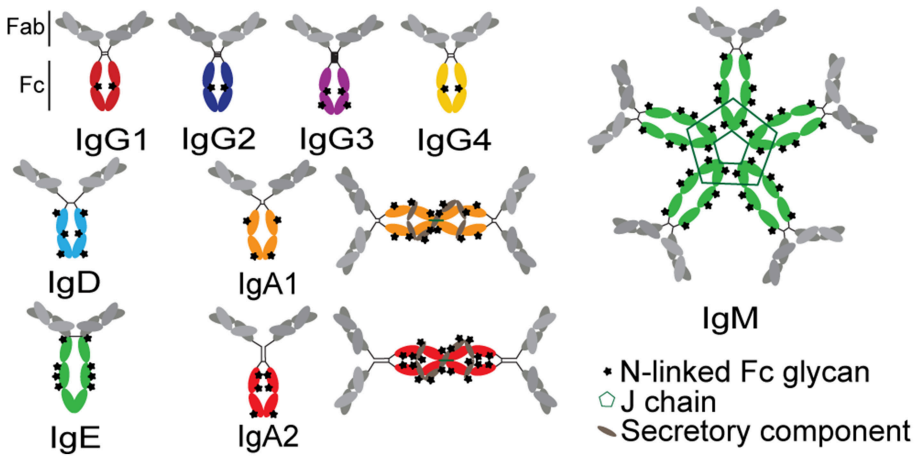

FIGURE 4 | Structures of antibody isotypes and subclasses. Fc domains are in color while Fab domains are in gray. Stars indicate N-linked Fc glycans. IgA isotypes are shown as both monomers (predominant in serum) and dimers (predominant at mucosal surfaces).

structure is biantennary, with a structure consisting of two-branched linked N-acetylglucosamine (GlcNAc), a mannose, followed by 2 branched mannoses, each followed by an additional GlcNAc on each mannose (**Figure 5**). Three additional sugars can then be added at variable levels, including a core fucose on the first GlcNAc, galactoses that can be added to each terminal GlcNAc, sialic acids that can subsequently be added to the each galactose, and finally the addition of a bisecting GlcNAc to the core mannose (**Figure 5**) (91). Given the variable addition of each of the 4 additional sugars, a total of 36 distinct glycan structures can be added to any given IgG (92). Importantly, while glycans do not interact directly with FcRs, they influence the flexibility and structure of the antibody Fc, thereby changing interactions with FcRs (93). Complete removal of the Fc glycan ablates low affinity FcR binding, with only high affinity FcγRI retaining measurable binding ability (94). Additionally, IgA and IgM antibodies are also Fc-glycosylated (**Figure 4**), although it is unclear how this glycosylation changes affinity for FcRs.

Across diseases, dramatic shifts have been identified in IgG glycosylation, such as a significant increase in agalactosylated antibodies in chronic inflammatory diseases such as autoimmune flares and HIV infection (95, 96). In the monoclonal therapeutics community, the systematic removal of specific components of the Fc N-glycan have highlighted the critical role of individual sugars in shaping antibody effector function (97). Specifically, the presence of sialylation drives anti-inflammatory activity *in vivo* (98, 99). The removal of fucose either directly or indirectly, through the upregulation of the bisecting GlcNAc, results in increased antibody affinity for FcγRIIIa and consequently enhanced ADCC (90, 100). Conversely, agalactosylated antibodies decrease ADCC and drive pro-inflammatory responses (90).

Beyond our emerging appreciation for a role of sialylated antibodies in vaccine induced affinity maturation (71, 101) described above, influenza vaccination is known to alter Fc glycosylation (102, 103). Soon after immunization, influenza-specific antibodies rose rapidly, and had increased galactosylation, increased sialylation, and reduced bisection compared to pre-existing influenza-specific antibodies (102, 103). However, these glycan shifts normalized after a month, suggesting that these transient changes in influenza-specific antibody glycan profiles may reflect differences in glycosylation by plasmablasts, not plasma cells. Even in the absence of vaccination, HA-specific antibodies exhibited unique glycan profiles compared to HIV-specific antibodies from the same individuals. Specifically, HA-specific antibodies were more highly galactosylated and sialylated and contained reduced b-GlcNAc (102), highlighting the unique glycan profiles that are naturally selected on influenza-specific antibodies. Whether individuals who control the virus more effectively tune antibody glycosylation in a specific or selective manner is unclear, but represents a simple strategy to modulate antibody function.

OPTIMIZING ANTIBODY RESPONSES THROUGH VACCINATION

Adjuvants

One of the greatest hurdles of influenza vaccination is overcoming response anergy caused by previous exposures to influenza in the aging immune system. An attractive strategy to overcome this anergy and generate protective humoral immunity, particularly for novel strains or universal vaccine formulations, is the use of adjuvants to enhance and tune the response to vaccination. There are currently four adjuvants licensed for use in influenza vaccines in the United States and/or in Europe: aluminum salts (alum), MF59, AS03, and virosomes (104). In addition to adsorbing antigens and creating multivalent lattices of antigens, alum activates the inflammasome promoting more effective responses upon antigen-presenting cell delivery (105). Alum induces primarily a Th2-driven response (104). However, Th1 responses are likely to be more critical in the clearance of intracellular pathogens, including influenza (105). Oil-in-water emulsions, such as MF59 and AS03, are

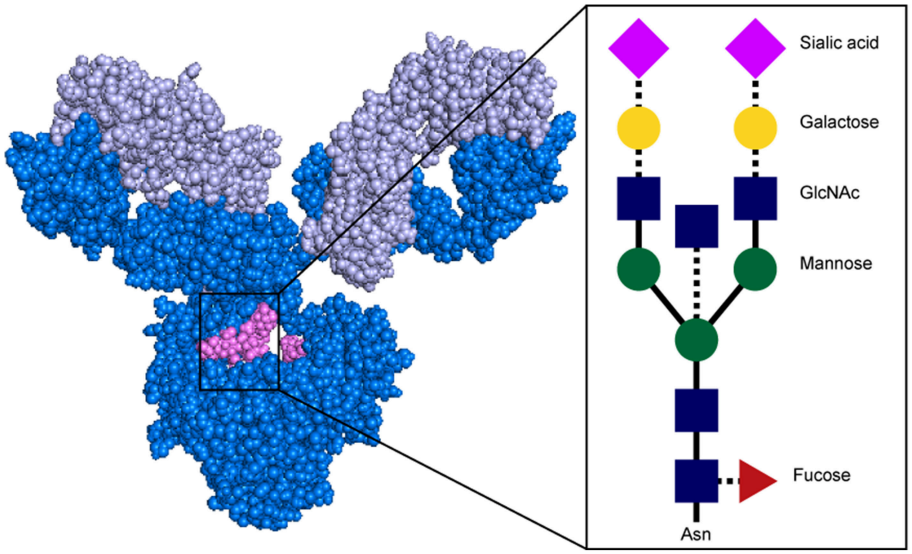

FIGURE 5 | Structure of antibody glycan. Antibody image shows heavy chain in dark blue, light chain in light blue, and glycan in magenta. In glycan schematic, solid lines indicate core glycan consisting of two-branched linked N-acetylglucosamine (GlcNAc; blue rectangle), a mannose (green circle), followed by 2 branched mannoses, each followed by an additional GlcNAc on each mannose. Dotted lines indicate additional sugars that can be added at variable levels, including a core fucose on the first GlcNAc (red triangle), galactoses (yellow circle) to each terminal GlcNAc, sialic acids (pink diamond) to the each galactose, and a bisecting GlcNAc to the core mannose. Antibody structure: PDB 1IGY.

predicted to work through a more balanced Th1/Th2 response, enhancing both T cell and antibody responses via delivery to antigen presenting cells as well as through the recruitment of specific innate immune cells to the site of injection (106). Specifically, in an adjuvanted HIV vaccine trial in macaques, MF59 increased recruitment of neutrophils, monocytes, and MDCs to the site of injection and recruitment of neutrophils to the draining lymph node (107). In the context of influenza vaccination, MF59 increased the heterosubtypic, or broadly reactive, antibody response and increased neutralizing antibody responses to influenza (108, 109). Unfortunately, MF59 also shifted the response even further toward the immunodominant HA head and away from the HA stem (110). Yet, MF59 increased the affinity of antibodies developed following both seasonal and novel pandemic influenza vaccines, suggesting that if skewed selectively to particular target antigenic sites, this adjuvant could drive enhanced affinity maturation to the correct sites of vulnerability (110). Virosomes or phospholipid vesicles, have also been studied in the context of influenza HA and NA vaccines, showing similar profiles to MF59 (104). The effects of these adjuvants on FcR-mediated antibody functionality are only beginning to be studied (107).

Other adjuvants are currently under investigation to specifically and selectively enhance influenza specific immunity. For example, liposomes provide unique scaffolds for antigen delivery (105), and were shown to increase the humoral and Th1 response, boosting neutralization, in mice following influenza vaccination (104). Additionally, virus-like particles, or nanoparticles, which deliver antigens in a multivalent manner, similar to their native conformation, increased heterosubtypic IgG2a neutralizing antibody titers in mice, the mouse analog of IgG3, the most functional antibody subclass in humans (111). Presentation of antigens in the form of a viral particle may play an essential role in driving functional antibody responses (112–114). Another type of adjuvant, ISCOMS (antigen, cholesterol, phospholipid and saponin-defined immunomodulatory complexes), created a balanced, protective immune response based on strong MHC class I presentation in trials with a pandemic influenza antigen (104). However, tests of ISCOMS with cancer antigens showed that this adjuvant shifted the response away from antibodies, toward CD4+ and CD8+ T cells, with limited changes seen to antibody responses (115). Finally, Toll-like receptor (TLR) agonists, involved in early pathogen sensing, are known to tune the inflammatory response to tailor immunity in a pathogen specific manner (104). Several TLR agonists were shown to increase influenza-specific antibody titers following vaccination, however their effects on antibody-mediated functions beyond neutralization are unexplored (104, 116). Thus, while previous studies with these adjuvants have primarily focused on neutralizing antibody responses, additional insights on the specific effects of adjuvants on shaping protective FcR activity will provide additional avenues to tune and direct protective immunity against influenza infection.

Antigen Design and Glycosylation

In addition to efforts to promote more effective immune stimulation through adjuvants, intense investigation has focused on the development and design of unique antigens able to selectively direct the immune response away from strain-specific immunodominant sites to those that are more conserved (17). These include the design of computationally enhanced globally

relevant HA sequences, the design of mini-antigens and chimeric antigens, and glycan-enhanced antigens.

Computationally optimized broadly reactive antigens (COBRAs), designed based on computational modeling of influenza strains to create mosaic antigens aimed at focusing the immune response on the evolution of heterosubtypic responses, had some success in eliciting broadly reactive HAI titers that target both seasonal and pandemic strains of influenza (117–120). In a recent study, COBRA H3s did not increase the breadth of HAI reactivity by vaccine-induced antibodies across a panel of strains. However, these COBRA H3s did increase the phylogenetic diversity of neutralized strains (120), meaning that the COBRA-induced responses altered which strains were recognized and neutralized without increasing the total number of strains recognized (120). These data suggest that COBRA antigens can increase heterosubtypic responses to conserved epitopes on the immunodominant head, but are unable to create broadly reactive responses to the conserved HA stem.

Given the complexity of altering immunodominance using whole HA molecules, additional efforts have aimed to direct immunity against minimal antigenic regions associated with broadly protective responses including the stem (121, 122) and the receptor binding site (123). Although broadly protective non-neutralizing responses can target the stem region of HA, these responses are typically subdominant (124). HA stem-only antigens or antigens with conserved stem domains but altered HA heads have been developed (17). For example, a "headless" HA vaccine tested in mice created a broadly protective non-neutralizing immune response (125). Furthermore, chimeric HA vaccines, which were used to immunize animals with "exotic" chimeric molecules that coupled unusual heads to a single stem region, have shown promise. Specifically, using heads that have not circulated in the population, coupled to conserved stems, this vaccine strategy drove robust focused stem-specific protective immune responses and higher cross-reactive HA-specific antibody titers, and are now in clinical trials (126, 127).

Seasonal influenza vaccines have been produced in eggs since the introduction of yearly vaccination, and the manufacturing techniques have remained largely unchanged for decades (128). Emerging data and technical advances are increasing the attractiveness of cell culture-based production strategies, rather than egg-based production. Beyond issues related to speed and cost of vaccine production across these platforms, qualitative differences in antigens from egg-based vaccines compared to circulating viruses may necessitate this shift. HA is highly N-glycosylated in a host-cell dependent manner (129–131). The glycosylation of egg-grown vaccine virus is different than that of naturally infecting virus (132). Emerging data points to the importance of glycosylation not only in shaping antigen-exposure on the surface of the HA molecule, such as masking of specific epitopes (131, 133), but also in contributing to the antigenicity of mAb binding epitopes (134–136). Differential HA glycosylation between egg- and cell culture-grown virus impacted innate immune interactions with the virus in the lung, including neutralization by surfactant protein D (SP-D) (137) and binding to mannose-specific lectins (138). Moreover, altered glycosylation was shown to change both cellular and humoral response kinetics *in vitro* and *in vivo* (131, 139). Vaccination of mice with de-glycosylated HA led to decreased CD4+ T cell activation and cytokine production, resulting in reduced HA-specific antibody titers and HAI titers (131, 139). Studies of the antibody response using differentially glycosylated (not de-glycosylated) HAs showed that glycosylation alters the binding and neutralization of monoclonal antibodies, but lacked further detail about the effects of glycosylation on polyclonal antibody pools or on Fc-mediated function (131, 139, 140).

Epidemiological studies in recent years investigating poor vaccine protective efficacy have shown that antigen glycosylation had a direct impact. In the 2016–2017 flu season, the circulating H3N2 virus had a new glycosylation site compared to previous seasons. However, the egg-adapted version of the viral strain used to produce the vaccine lacked this site through an amino acid mismatch in an antigenic site, resulting in decreased vaccine effectiveness (134). Given that glycosylation can strongly impact epitope antigenicity, a vital mismatch at a site of neutralization sensitivity resulted in the induction of non-protective immunity and rendered the circulating virus invisible to vaccine induced immune responses. Shaping glycosylation to produce representative antigens is critical to achieving protective immune responses to vaccination.

Additional Antigenic Targets

While the majority of the humoral immune response is directed toward the immunodominant HA molecule, antibodies also emerge against other targets including neuraminidase (NA), nucleoprotein (NP), and Matrix-2 (M2) (**Figure 1**). Antibodies targeting NA, while not neutralizing, can prevent viral exit from infected cells to block subsequent rounds of infection (12), and have been associated with seasonal protection (12, 141–143). However, NA-specific antibodies have also been shown to drive ADCC (51, 144), suggesting that this less immunodominant target is vulnerable to multiple modes of antibody mediated targeting.

The highly conserved internal viral proteins NP and M2 have been shown to induce an immune response that is also broadly reactive (145). NP-specific antibodies, which are always non-neutralizing (146), mediated viral clearance through FcRs and protection in mouse models of influenza infection (143, 147). Similarly, non-neutralizing M2-specific antibodies mediated ADCC and ADCP to clear infected cells and promoted rapid viral clearance (141, 142). Thus, while current vaccination strategies largely focus on the development of broadly reactive immunity against HA, additional largely non-neutralizing conserved antigens exist within influenza that may represent next generation targets for protective universal immunity.

CONCLUSION

More than 4 decades of research has clearly illustrated the importance of both direct neutralization and non-neutralizing functional antibodies in protection against influenza infection and disease. Because neutralizing and non-neutralizing antibody

activities are not induced in a mutually exclusive manner, vaccine strategies able to leverage both functions of antibodies are likely to confer the greatest level of protection. However, the precise innate immune effector functions to precise sites of viral vulnerability on HA or other target antigens remain to be determined. With emerging novel vaccine design strategies, coupled to emerging immune modulatory adjuvants, opportunities to drive universal protection are on the horizon.

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CB and GA wrote the review article, contributed to revision, and approved the submitted version.

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REVIEW
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The Human FcγRII (CD32) Family of Leukocyte FcR in Health and Disease

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FcγRs have been the focus of extensive research due to their key role linking innate and humoral immunity and their implication in both inflammatory and infectious disease. Within the human FcγR family FcγRII (activatory FcγRIIa and FcγRIIc, and inhibitory FcγRIIb) are unique in their ability to signal independent of the common γ chain. Through improved understanding of the structure of these receptors and how this affects their function we may be able to better understand how to target FcγR specific immune activation or inhibition, which will facilitate in the development of therapeutic monoclonal antibodies in patients where FcγRII activity may be desirable for efficacy. This review is focused on roles of the human FcγRII family members and their link to immunoregulation in healthy individuals and infection, autoimmunity and cancer.

Keywords: Fc receptor, FcγR, inflammation, infection, autoimmunity, cancer, mAb therapeutics

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INTRODUCTION

Fc receptors are, by definition, receptors for the Fc portion of immunoglobulins (Ig). These have been traditionally viewed primarily as cell surface receptors for Ig and whose interaction drives a surprisingly diverse range of responses mostly within the immune system or related to the physiology of antibodies in immunity.

Receptors for IgM, IgA, IgG, and IgE have been defined over the last 40 years with the majority of research focused on the receptors found on leukocytes. These receptors induce or regulate leukocyte effector functions during the course of immune responses. It is noteworthy, and also beyond the scope of this review, that a limited number and type of Fc receptors are also expressed on cells outside the immune system where they affect or participate in physiology of antibody function.

In humans, the largest grouping of Fc receptors is the "leukocyte Fc receptors" expressed primarily on effector cells. Their ectodomains bind ligand, the IgG antibody Fc region, and belong to the Ig-superfamily. They include the high affinity IgE receptor FcεRI and the distantly related IgA receptor FcαRI, but the largest group are the IgG receptors or the FcγRs which themselves comprise several groups—FcγRI, the high affinity IgG receptor, the FcγRII family (FcγRIIA, FcγRIIB, FcγRIIC), and the FcγRIII family (1, 2).

THE HUMAN FcγRII (CD32) FAMILY OF LEUKOCYTE FCR

General Comments

The human FcγRII family (also known as CD32 in the Cluster of Differentiation nomenclature) consists of a family of primarily cell membrane receptor proteins. They are encoded by the mRNA splice variants of three highly related genes—*FCGR2A, FCGR2B,* and *FCGR2C,* which arose by recombination of the *FCGR2A* and *FCGR2B* genes (3).

All members of the FcγRII family are integral membrane glycoproteins and contain conserved extracellular domains, exhibiting an overall 85% amino acid identity (3, 4). The high degree of amino acid and DNA identity has posed challenges in the analysis of receptor function using monoclonal antibody or nucleic acid based methods. Thus, some caution should be exercised when analyzing literature or interpreting experimental data. The encoded products of the three genes are low-affinity receptors that are defined practically as interacting poorly with monomeric IgG, i.e., micromolar affinity (5, 6), but when arrayed on the cell surface, they avidly bind multivalent complexes of IgG, e.g., immune complexes.

The FcγRIIA (also FcγRIIC) and FcγRIIB proteins have opposing cellular functions. FcγRIIA proteins are activating-type Fc receptors. In contrast, FcγRIIB is a key immune checkpoint that modulates the action of activating-type Fc receptors and the antigen receptor of B cells. When expressed, the FcγRIIC proteins retain the activating function of the cytoplasmic tail of FcγRIIA and the binding specificity of FcγRIIB ectodomains.

The focus of this review is the FcγRII family and their actions as receptors for immunoglobulins. It should be noted that FcγRIIA also acts as a receptor for pentraxins, a product of innate immunity that is important in infection and inflammation and which has been recently reviewed elsewhere (7). Since much of the biology of the Fc receptors has been determined in the mouse, it is noteworthy that the human and mouse FcR families differ significantly, with FcγRIIB being the only FcγRII forms in the mouse. Also, although the human and mouse FcγRIIB homologs are highly conserved, there are differences in their splice variants in the two species (see below). Importantly, cellular expression can also vary between humans and mice.

Human FcγRII gene polymorphism, mRNA splicing, and copy number variation (CNV) further diversifies the potential biological consequences of IgG interactions with the FcγRII receptor proteins. These properties and roles of each group of FcγRII proteins are reviewed in detail in the following sections.

PROPERTIES OF FcγRIIA

Molecular Structure

The human FcγRIIA proteins were originally defined by cross-species gene cloning (8). They are encoded by the *FCGR2A* gene (**Figure 1**) and are comprised of eight exons; two encoding the 5‘ UTR, and leader sequence and the N-terminus of the mature protein; one exon for each of the two Ig-like domains of the extracellular region; one exon for the transmembrane domain; and three exons encoding the cytoplasmic tail and 3′ UTR (3). Three mRNA transcripts, two of which encode membrane proteins, arise by alternative splicing of the mRNA (**Figure 1**).

The most extensively characterized form is the canonical 40 kDa integral membrane protein, FcγRIIA1, that contains all but the first (C1*) cytoplasmic sequence (3, 4, 8–10). A second, but relatively rare, membrane form has been recently described (11, 12). FcγRIIA3 is identical in sequence to the canonical FcγRIIA1, with the notable exception of a 19-amino acid insert in its cytoplasmic tail, arising from the inclusion of the C1* exon which was believed previously to be a vestigial or cryptic exon (4). This insertion is highly homologous (18/19-amino acids) to the insertion present in the cytoplasmic tail of inhibitory FcγRIIB1 (11–13). mRNA splicing that successfully gives rise to FcγRIIA3 is associated with an *FCGR2A*$^{c.7421871A>G}$ SNP that creates a splice acceptor site, which greatly increases the inclusion of the C1* exon (11).

An unusual mRNA has been reported that lacks the transmembrane exon resulting in a potentially secreted 32 kDa polypeptide (14). This FcγRIIA2 form is not extensively characterized and its physiology is uncertain. However, it raises the possibility that naturally occurring soluble forms may act as modulators of immune complex-induced activation and inflammation and it is noteworthy that recombinant soluble FcγRIIA inhibits immune complex-induced activation of inflammatory cells *in vitro* and *in vivo* (9).

Cellular Expression

The FcγRIIA proteins are unique to primates (15, 16). FcγRIIA1 is the most widespread and abundant of all FcγR, present on Langerhans cells, platelets and all leukocytes, with the exception of most lymphocytes (**Table 1**) (1, 16, 17). FcγRIIA3 is expressed by neutrophils and monocytes (11) and FcγRIIA2 mRNA is present in platelets, megakaryocytes, and Langerhans cells (14). The levels of FcγRIIA expression are influenced by cytokine exposure. Interferon (IFN)-γ, interleukin (IL)-3, IL-6, IFN-γ, C5a, prostaglandin-E (PGE), and dexamethasone increase expression, but IL-4, tumor necrosis factor (TNF)-α, and TNF-β reduce expression (18–21). There are also reports of FcγRII induction on CD4 and CD8 T cells upon mitogen or TCR stimulation. Both FcγRIIA and FcγRIIB are reported to be expressed on activated CD4 T cells (22, 23).

FcγRIIA Signaling ITAM Activation vs. ITAM Inhibition

Like other activating-type immunoreceptors, FcγRIIA and FcγRIIC signal via the Immunoreceptor Tyrosine-based Activation Motif (ITAM) pathway (24–26) with a major structural difference. In the case of all other activating-type immunoreceptors—which includes the antigen receptors as well as the activating type FcR, e.g., FcεRI, FcγRIIIA—the ligand binding chain and the signaling subunits are encoded in separate polypeptides e.g., FcγRIIIA and the common FcR-γ chain dimer. The assembly of a functional signaling complex requires their non-covalent association (17). However, in the case of FcγRIIA and FcγRIIC, the ITAM is present in its own IgG binding chain. Furthermore, the FcγRIIA ITAM is unusual in that it does not fit the canonical ITAM consensus sequence and includes three additional aspartic residues (**Table 2**), although how this affects FcγRII function remains unknown (13). ITAM signaling is essential for FcγRIIA-dependent phagocytosis and the induction of cytokine secretion induced by its aggregation by immune complexes. Such high stoichiometry aggregation of receptors results in receptor-associated src family kinase, particularly Fyn (27), mediated phosphorylation of the two tyrosines of the ITAM and the recruitment of Syk and the propagation of activatory signaling pathways. In human FcγRIIA transgenic mice, Fyn deficiency is protective in models of FcγR dependent nephritis

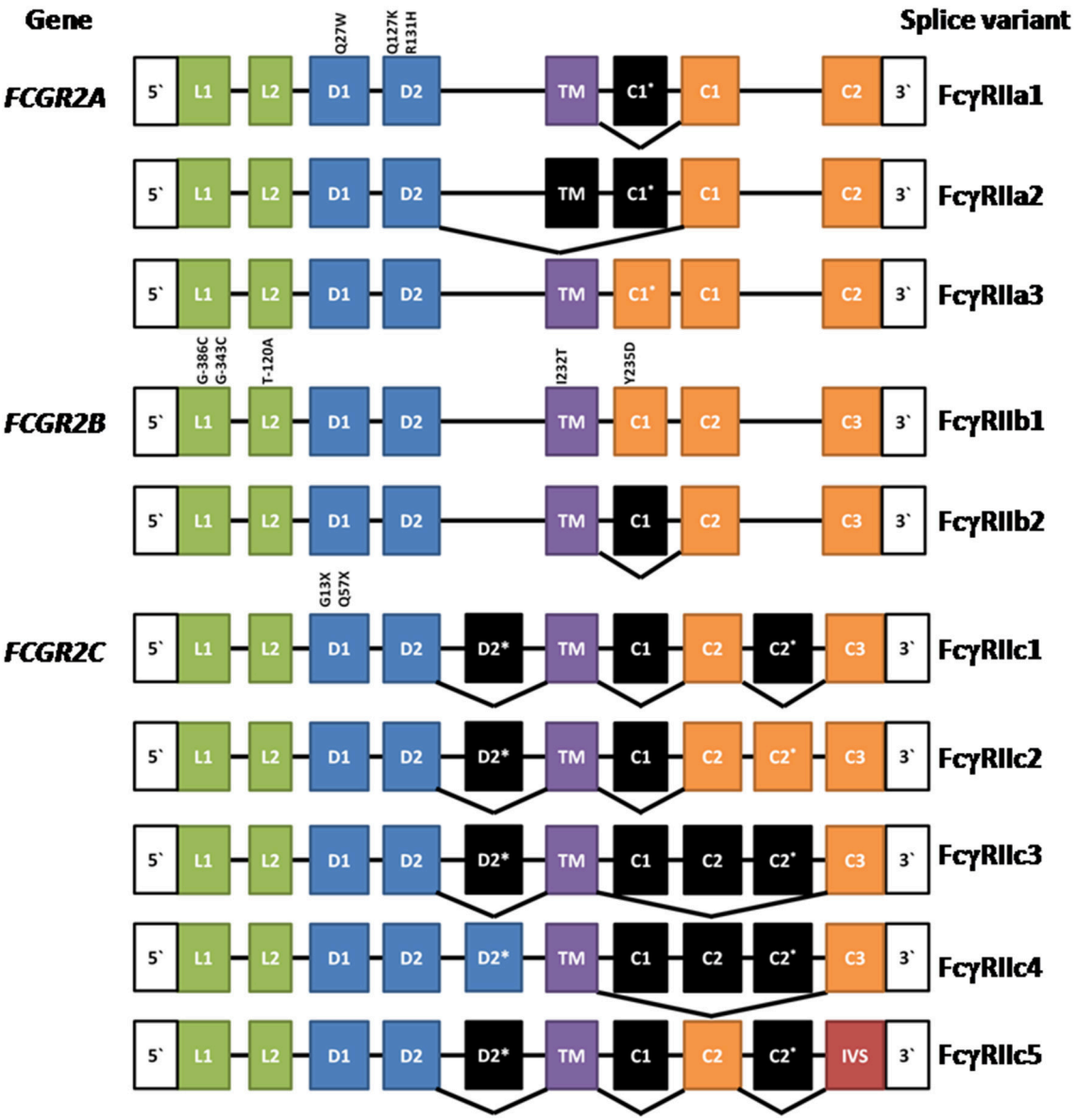

FIGURE 1 | Composition of *FCGR2A*, *FCGR2B,* and *FCGR2C* and their splice variants. Leader (L), ectodomain (D), transmembrane (TM) cytoplasmic tail (c), and intervening sequence (IVS). Expressed exons are illustrated in color, while spliced exons (selectively expressed) are represented in black. The location and position number of amino acids affected by well characterized polymorphisms are shown above the exons except for the *FCGR2B* leader exons where the nucleotide positions are given. See text for references.

and arthritis, indicating a pivotal pro-inflammatory role for Fyn kinase in ITAM signaling (27).

Since the original characterization of the activating role of ITAM pathway was described, it is now apparent that ITAMs can under certain circumstances mediate inhibitory or modulating function termed ITAMi (inhibitory ITAM) (28, 29). Under conditions of low stoichiometric interaction, the receptor-associated src family kinase Lyn phosphorylates only one of the two tyrosine residues (mono-tyrosine phosphorylation) within the ITAM, with two juxtaposed receptors presenting mono-phosphylated-ITAMs to recruit the two SH2 domains of the SH2-domain containing protein tyrosine phosphatase 1 (SHP-1). This interaction is not dissimilar to SHP1 binding via its dual SH2 domains to inhibitory immunoreceptors with dual ITIMs (30). Then Lyn phosphorylation of Tyr^{536} of SHP-1 positively regulates SHP-1 phosphatase activity resulting in the inhibition of cell activation (27). Animal studies suggest that the ITAMi effect ameliorates pathological inflammatory responses and may also be important in controlling "baseline" receptor activation. This ITAMi effect is not unique to the unusual

TABLE 1 | Leukocyte Expression of FcγRII forms.

Cell type	FcγRIIA	FcγRIIB	FcγRIIC[a]
T cells	¡[b]	¡[b]	?
B cells	−	+++	+
NK cells	−	−[c]	+
Macrophages	+++	++	?
Monocytes	+++	+	?
Neutrophils	+++	+	?
Eosinophils	++	•	•
Basophils	++	+++	−
Mast cells	++	−[d]	−
Platelets	++	−	−

+++ High, ++ Moderate, + Low, or − No expression. • no data.
[a]Expressed only in ~20% of humans;
[b]Expression induced in some T cell subpopulations;
[c]Expressed as a result of promoter modification related to FcγRIIC allelism.
[d]Conflicting results.

TABLE 2 | Sequence comparison of ITAMs of activating type FcγR.

Receptor ITAM	Consensus[a]
FcR-γ chain	**Y**TG**L** STRN− − −QET **Y**ET**L**
FcγRIIA and Fcγ IIC	**Y**MT**L** NPRAPTDDDKNI **Y**LT**L**

[a]Bold letters in FcRγ chain and FcγRIIA sequences indicate the critical Tyr and Leu residues of the ITAM consensus motif YxxL/I (6–12) YxxL.

FcγRIIA ITAM (29) as it has been also described for FcαRI (31, 32) and FcγRIIIA (33), both of which signal through the common FcR-γ chain dimer which contains canonical ITAMs.

Cellular Responses

FcγRIIA aggregation by IgG cross-linking initiates a variety of effector responses, depending on cellular expression which is affected by the local cytokine environment, and cross-talk between other FcR and TLR (34, 35). Internalization via both endocytosis and phagocytosis can be mediated by FcγRIIA in cell lines, i.e., ts20 (36, 37), COS-1 (38), U937 (39) as well as in primary human cells i.e., neutrophils (40, 41), monocytes (40), platelets (40, 42), and macrophages (43). FcγR phagocytosis requires ITAM activation, which also initiates the ubiquitin conjugation system. Conversely, endocytosis is dependent only on ubiquitination and clathrin, not ITAM phosphorylation (36, 37).

The internalization of antigen: antibody immune complexes by FcγR on antigen presenting cells (especially dendritic cells) is an important part of antigen presentation for the development of effective immune responses. This process also increases the efficiency of T cell activation particularly in response to low concentrations of antigen (44). The role of human FcγR in antigen presentation is well documented in *in vitro* systems and it appears that all FcγR are important at some level (45–47). However, more recent analyses have shown FcγRIIA is the major receptor in the development of so-called "vaccinal effects" of monoclonal antibody therapy in cancer. It appears that the therapeutic antibodies targeting cancer cells can induce a long lasting protective response beyond the acute therapeutic phase of the therapy (48).

FcγRIIA1 activates neutrophils and other myeloid effector cells for direct killing of IgG-opsonized target cells including tumor cells and virus-infected cells (49). Also, FcγRIIA binding of IgG immune complexes triggers granulocytes to release inflammatory mediators such as prostaglandins, lysosomal enzymes, and reactive oxygen species, as well as cytokines including IFNγ, TNFα, IL-1, and IL-6 (50, 51). The FcγRIIA3 splice variant form is an even more potent activator of human neutrophils than FcγRIIA1, and is responsible for some severe adverse reactions to immunoglobulin replacement therapy (11). The mechanistic basis of this potency relates to its longer retention time in the cell membrane and the consequential enhanced ITAM signaling (12). Whilst this enhanced potency may present a risk factor for hypersensitivity to immunoglobulin replacement therapy, it may provide some benefit for protection against infection.

The limited number of studies of FcγRII expression of human T cells suggest FcγRII crosslinking on TCR-stimulated CD4 T cells enhances proliferation and cytokine secretion, suggesting an activating function of FcγRIIA (22, 23). The nature of FcγRIIA expression on CD4 T cells is not straightforward nor completely characterized. Purified CD4 T cells when stimulated with anti-CD3/CD28 induced surface expression of FcγRII on 10% of cells and intracellular expression in 50%. In contrast, unstimulated cells express little FcγRII (23). Imaging of FcγRII-expressing CD4 T cells sorted from unstimulated normal peripheral blood mononuclear cells, or those from HIV-1$^+$ individuals shows cells displaying punctate FcγRIIA staining (23) or discrete patches of B cell membrane. These B cell membrane patches include FcγRIIB and CD19 markers (52), consistent with possible trogocytosis by the activated T cell from the B cell. Similarly, FcγRIIIA is also expressed on activated CD4 T cells, and this expression appears to be both intrinsic upon cell activation and acquired by trogocytosis of APC membrane (53).

FcγRIIA plays an important role in the normal physiology of platelet activation, adhesion, and aggregation following vessel injury (54). More recent studies indicate FcγRIIA associates with glycoprotein (GP) Ib-IX-V on platelets and can thereby be indirectly stimulated by von Willebrand factor (VWF) or after stimulation of G-protein-coupled receptors (GPCRs) (54). Interestingly, FcγRIIA signaling on platelets is regulated by proteolytic cleavage of the cytoplasmic tail, or "de-ITAM-ising" (55).

PROPERTIES OF FcγRIIB

Molecular Structure

Initially, FcγRIIB was discovered in the mouse by protein sequence and molecular cloning analyses (56, 57) and the human *FCGR2B* gene was then isolated by cross species hybridization. Human *FCGR2B* has similar structure to human *FCGR2A,* being comprised of eight exons. The two major forms of FcγRIIB—FcγRIIB1 and FcγRIIB2 (**Figure 1**)—arise from mRNA splicing

which results in the inclusion or exclusion of the C1 exon sequence in FcγRIIB1 and FcγRIIB2 isoforms, respectively (3, 4). The inclusion of the C1 exon sequence in the FcγRIIB1 results in tethering to the membrane of B cells, whereas its absence from FcγRIIB2 allows rapid internalization of the receptor in myeloid cells. Both forms contain the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in their cytoplasmic tails. The extracellular domains are 95% identical to the two domains of FcγRIIA and almost completely identical to the FcγRIIC (3, 8, 17). Although the focus of this review is the human FcγRII, it should be noted that mouse FcγRIIB comprises three splice variants FcγRIIB1, FcγRIIB1′, and FcγRIIB2, with the predicated amino acid sequences of the latter two corresponding to the human FcγRIIB1 and FcγRIIB2 variants. Any functional differences between the two mouse FcγRIIB1 and FcγRIIB1′ forms are unknown (58). There are also amino acid sequence differences between human FcγRIIB1 and mouse FcγRIIB1/1′ and the functional consequences of these are also unknown.

Cellular Expression

As indicated in "General Comments" above, the analysis of expression of human FcγRIIB protein has been historically difficult because of the extremely high sequence conservation of the extracellular domains of FcγRIIB, FcγRIIA, and FcγRIIC and lack of specific monoclonal antibody probes. The high degree of DNA sequence conservation has also confounded analysis. Much of the early literature has relied on either PCRs or interpretation of data using antibodies that are cross-reactive with, or specific for, FcγRIIA or a combination of these methods and reagents. The relatively recent development of such FcγRIIA/C and FcγRIIB specific antibodies (59–61) has now helped to clarify expression patterns, but there are still differences reported between groups using these reagents. Some caution should still be exercised in analysis of the historic literature. Furthermore, cell expression patterns of FcγRIIB in mouse myeloid derived cells is substantially different to human FcγRIIB, thus additional caution is advised in interpreting the data. Nonetheless, it is clear that FcγRIIB (FcγRIIB1) is highly expressed by B cells, and its mRNA has also been identified at lower levels on monocytes (**Table 1**) (62). The levels of FcγRIIB expression are influenced by cytokine exposure. Cytokines such as IL-10, IL-6, and dexamethasone increase expression of FcγRIIB, while TNF-α, C5a and IFN-γ inhibit expression (18–20).

FcγRIIB (FcγRIIB2) is highly expressed on basophils and at low levels on monocytes (63). Expression on other granulocytes is somewhat complex and controversial. The differences in reported expression of FcγRIIB on mast cells may reflect technological limitations or differences in tissue origin of the cells under investigation. Intestinal and cord blood derived mast cells have been reported as expressing FcγRIIB on the basis of mRNA expression (64). In one study using human leukocyte reconstituted mice and a FcγRIIB specific polyclonal antibody, FcγRIIB protein was detected (65). However, skin mast cells lack FcγRIIB surface expression (66) and using a FcγRIIB specific mAb, peripheral blood derived mast cells do not express FcγRIIB (A. Chenoweth personal communication). Neutrophils either lack (60) or express very low levels of FcγRIIB (59), and the FcγRIIB-specific mAb 2B6 does not usually stain NK cells (60). However, in that proportion (∼20%) of the population where FcγRIIC is expressed, NK staining by FcγRIIB antibodies might be expected as FcγRIIC EC domain is identical to FcγRIIB. A further complication is that FcγRIIC CNV affects control elements of the *FCGR2B* gene permitting FcγRIIB expression in NK cells (67) (see FcγRIIC below).

One of the more interesting features of FcγRIIB is its presence on non-leukocyte cells including airway smooth muscle (68) and liver sinusoidal endothelial cells (69). Its abundance in liver, in the mouse, accounting for three quarters of the total body expression, appears to provide a large sink for the removal in IgG immune complexes, which has been exploited in therapeutic monoclonal antibodies whose Fc portions have been engineered for high affinity binding to FcγRIIB (70, 71). This appears to be a "stand alone" function of FcγRIIB where small immune complexes are internalized without risk of pro-inflammatory activation.

FcγRIIB Modulation of Immunity

FcγRIIB was the first immune "checkpoint" defined (72), with mouse studies showing a pivotal role in controlling autoreactive germinal center B cell activation and survival in mice with dysfunction resulting in loss of tolerance and autoimmunity (73, 74). Mice with humanized immune systems reconstituted with stems cells homozygous for the dysfunctional FcγRIIB Thr^{232} allele develop autoantibodies with specificities characteristic of lupus and human rheumatoid arthritis (75). This critical action of its ITIM in controlling the ITAM activation pathway is extensively reviewed elsewhere (25, 76). The ratio of activating vs. inhibitory receptors is a key factor in determining the cellular threshold for cell activation and resulting immune response (18, 77). An ITIM, consensus amino acid sequence YXXL (where X represents any amino acid), is found in the cytoplasmic domains of both FcγRIIB1 and FcγRIIB2. The co-engagement of FcγRIIB with an activating type receptor such as FcγRIIA or the B cell antigen receptor (25) modulates their ITAM-mediated activation signal. FcγRIIB expression on innate effector cells modulates cell activation mediated by activating FcγRs, including dendritic cell maturation and antigen presentation. FcγRIIB also regulates signaling from varied innate cell receptors including TLRs and complement receptors, reviewed in Bournazos et al. (34) and Espeli et al. (78).

Much of the detail in understanding of the ITIM:ITAM system of immune cell modulation has been derived from FcγRIIB1 ITIM-mediated regulation of the B cell receptor (BCR) signaling in mouse B cells. Conventional FcγRIIB-mediated inhibition requires ligand-dependant co-engagement/aggregation of ITAM-containing receptors (79, 80). The FcγRIIB ITIM modulation targets the two major ITAM driven pathways—ITAM tyrosine phosphorylation, and the generation of phospholipid mediators, e.g., Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Briefly, src kinases such as Lyn kinase, which participate in the phosphorylation of the ITAM of the ligand-clustered activating receptors, also phosphorylate the FcγRIIB ITIM of the co-aggregated inhibitory receptor. Notably, FcγRIIB1 has been reported to be phosphorylated by Lyn and Blk, whereas FcγRIIB2 solely by Blk (81).

The phosphorylated-ITIM of FcγRIIB recruits the inositol phosphatases SHIP1 and SHIP2, as is extensively reviewed in Getahun and Cambier (25). The preferential recruitment of SHIP, over SHP1 and SHP2, to the phosphorylated FcγRIIB cytoplasmic domain is determined by the SHIP SH2 domain's affinity for the pITIM (82). Notably studies of SHIP recruitment to the cytoplasmic domain of mouse FcγRIIB1 found phosphorylation of Tyr^{326}, outside the ITIM, bound the SH2 domain of the adaptor Grb2 which bridged and stabilized the FcγRIIB:SHIP complex (83). Human FcγRIIB lacks an equivalent tyrosine, and has a small adjacent deletion. It fails to recruit Grb2 but still recruits SHIP1 that modulates BCR-induced Ca mobilization (84). SHIP dephosphorylates phosphatidylinositol species, with the predominant *in vivo* substrate being phosphatidylinositol 3,4,5-trisphosphate and ultimately recruits p62 Dok to form a highly active membrane localized enzymatic complex. This inhibits the Ras activation pathway, decreases MAP kinase activation and reduced PLCγ function leads to less activation of PKC. SHIP-dependent ITIM inhibition of the MAP kinase pathway, together with the anti-apoptotic kinase Akt can thereby affect cellular proliferation and survival (25).

The same mechanisms defined for BCR regulation are applicable to human and mouse myeloid cells, where many observations have been confirmed, particularly for FcγRIIB2 regulation of FcεRI (25, 76). Overall FcγRIIB1 and FcγRIIB2 signaling pathways are similar, however their principal functional difference lies in their localization in the cell membrane. The C1 insertion (85) of FcγRIIB1 prolongs membrane retention, whereas FcγRIIB2 is rapidly internalized. The equivalent C1* sequence in FcγRIIA3 also alters membrane localization (see above).

An ITIM independent mechanism of B cell regulation by FcγRIIB has been reported wherein FcγRIIB, by binding antigen bound IgG, co-aggregates with the BCR and prevents the membrane organization of BCR and CD19 (86, 87). In another mode of regulation of the adaptive humoral response, FcγRIIB has been reported to be expressed on plasma cells and binding IgG immune complexes and trigger apoptosis (88). Studies have also identified other mechanisms of FcgRIIB modulation of the IgE receptor and the BCR the existence of which in human cells has not been determined. Mouse bone marrow derived mast cells, which differ phenotypically from human mast cells, showed an unconventional FcγRIIB ITIM-dependent regulation of the high affinity IgE receptor, FcεRI, where intracellular mediated co-aggregation of FcεRI with FcγRIIB occurs independently of the FcγRIIB ectodomain binding to antigen complexed IgG (89).

Cellular Responses

The specific effects of FcγRIIB signaling are dependent on the context of the co-engaged activating receptors and the cell type. In B cells, FcγRIIB1 inhibition of the BCR is a critical immune checkpoint for regulating antibody production (25, 90). The powerful nature of this immune checkpoint is evident from studies in clinical, genetic, and animal models that show that altering the balance between ITIM modulation and ITAM activation is central to the pathogenesis and severity of disease (91).

As humoral immune responses develop, circulating antigen:antibody complexes simultaneously engage the antigen-specific BCR via the antigen of the complex and FcγRIIB via the Fc region, thereby modulating antigen receptor signaling. In FcγRIIB1, the C1 insertion impairs endocytosis, increasing the interaction time between FcγRIIB1, and the BCR. The C1 insert, irrespective of its position in the cytoplasmic tail, tethers the receptor to the cytoskeleton and so prevents the receptor localizing to coated pits and so disrupting endocytosis (92, 93). A di-leucine motif within the FcγRIIB ITIM sequence is also required for endocytosis (93, 94). Thus, the C1 insert confers cytoskeletal tethering and membrane retention which counter other cytoplasmic tail sequences including the di-leucine residues that would otherwise promote endocytosis.

FcγRIIB2 has also been studied in B cells in experimental systems where it also co-engages the BCR and regulates its function. FcγRIIB2 lacks the cytoplasmic C1 insertion and is rapidly internalized. A rare $Tyr^{235}Asp$ polymorphism occurs within the unique membrane-tethering 19-amino acid insertion of FcγRIIB1. FcγRIIB1-Asp^{235} binding of mouse IgG1 was slightly lower in comparison to the Tyr^{235} variant of FcγRIIB1, as was mIgG1 anti-CD3 induced T cell mitogenesis (95, 96). FcγRIIB1-Asp^{235} retained the capacity to form caps and was effective in down-regulating increases in calcium upon cross-linking by serum IgG (95).

This prolonged surface expression of actively signaling FcγRIIB1 may also be important for the elimination by apoptosis of self-reactive B cells during somatic hyper-mutation (97). Thus, FcγRIIB1 constrains the selective antigen specificity of the humoral immune system and directs the B cell production toward an appropriate antibody repertoire.

FcγRIIB is upregulated after antigen stimulation via immune complexes on follicular DCs (FDCs) (98). FDCs retain immune complexes and recycle them periodically to their plasma membrane, a process believed to be important in development of B cell immune cell memory (99). The presentation of immune complexes by activated FDCs expressing FcγRIIB provides antigens to B cells in a highly immunogenic form by multimerising the antigens, thus extensively crosslinking multiple BCRs, minimizing B cell FcγRIIB ITIM-mediated inhibition and providing co-stimulatory signals (100).

The functional response of a cell that expresses both ITAM-bearing receptors and FcγRIIB can be altered by their expression levels. Basophils express activatory FcεRI and FcγRIIA, as well as FcγRIIB, which can inhibit IgE-induced responses (101, 102). This balance can be altered by IL-3 which upregulates expression of both FcγR, but more strongly enhances FcγRIIB2 expression (101). Under normal physiologic conditions it is believed that FcγRIIA co-aggregation may, by providing activated Lyn, aid FcγRIIB inhibitory function (102).

Monocyte-derived dendritic cells (moDCs) that were treated with IFNγ to upregulate their activating FcγRs (FcγRI and FcγRIIA) had increased IgG-mediated cellular maturation, while moDCs treated with anti-inflammatory concentrations of soluble monomeric IgG (IVIg) to increase FcγRIIB expression had

decreased cellular maturation (18). Similarly, monocytes with increased expression of activating FcγRs over FcγRIIB as induced by IFNγ or TNFα had enhanced IgG-triggered cytokine production, while monocytes with enhanced FcγRIIB expression by IL-4 and IL-10 prevented IgG-triggered cytokine production (103). Furthermore, FcγRIIB$^{-/-}$ mouse macrophages developed robust inflammatory responses after exposure to subthreshold concentrations of immune complexes that failed to induce responses in FcγRIIB-expressing cells, demonstrating a role of FcγRIIB in setting a "threshold" for cellular activation (104).

PROPERTIES OF FcγRIIC

Molecular Structure

The expression of the membrane *FCGR2C* is complex. It is subject to a polymorphism (Gln^{13}STOP) wherein ∼80% of the population do not express functional FcγRIIC proteins and also CNV, which in turn impacts expression of the *FCGR2B* gene as described above (67, 105). The *FCGR2C* gene arose by recombination between *FCGR2B* and *FCGR2A*. The functional transmembrane FcγRIIC protein encoded by this gene is an activating receptor wherein the extracellular domains are derived from and are identical to FcγRIIB (exons 1–4), but the transmembrane and cytoplasmic tail are derived from the activating type ITAM-containing FcγRIIA (exons 5–8).

Multiple mRNA splice variants of FcγRIIC have been identified (**Figure 1**), though their physiology is unclear. Interestingly, some FcγRIIC-Gln^{13} individuals still lack FcγRIIC expression due to alternative splicing that gives rise to multiple non-functional forms (67). Additionally, the *FCGR2C* locus shows CNV, which may contribute to variation in gene expression, at the transcript and/or protein level, also impacting other FcγRII expression and function (67, 106).

Expression and Cellular Responses

In individuals expressing the activatory FcγRIIC, it has been most extensively studied on NK cells (**Table 1**). NK cells expressing FcγRIIC had increased levels of ADCC upon receptor cross-linking, causing mediator release and lysis of target cells (67, 105–108). Although not extensively studied, it appears that FcγRIIC is also expressed on CD19+ B Cells. Its co-ligation with the BCR caused enhanced BCR signaling and B cell function, relative to FcγRIIB ITIM-dependent negative regulation in the absence of FcγRIIC. This FcγRIIC expression on B cells is associated with systemic lupus erythematosus (SLE) in humans, possibly related to the altered or unbalanced ITAM/ITIM signaling (108).

Interestingly, multiple other SNPs, 114945036, rs138747765, and rs78603008, have been significantly associated with FcγRIIA or FcγRIIC mRNA expression in B cells in European populations (109). However, protein expression data is not yet available.

STRUCTURAL BASIS OF FcγRII INTERACTION WITH IgG

Human FcγRs have distinct binding specificities and affinities for the four IgG subclasses (2). The determination of affinity and IgG subclass specificity has relied on a wide range of methods mostly based on the binding of immune complexes to cell-expressed FcγR. More sensitive methods have used recombinant ectodomains and monomeric IgG using highly sensitive cell free systems such as SPR (5, 6, 110). A survey of the literature on the measurement of specificity and affinity of these receptors shows some variation in the methods used and the values calculated. Even the application of more sophisticated methods such as SPR show some degree of variation from group to group. Notwithstanding the variations and limitation in analyses of the interactions, it is clear that the FcγRII family (FcγRIIA, FcγRIIB, and FcγRIIC), are sensors of immune complexes and as such, interact poorly with uncomplexed monomeric IgG (1 μM affinity) but avidly bind immune complexes (5, 6, 15, 110).

TABLE 3 | Relative binding of human IgG by FcγR expressed on the cell surface.

Human FcγR	Human IgG Subclass			
	IgG1	IgG2	IgG3	IgG4
FcγRIIA His^{131}	+++	++	++++	−
FcγRIIA Arg^{131}	++	±	++++	±
FcγRIIB	+	−	+++	+
FcγRIIC	+	−	+++	+

There is general agreement that all FcγRII, indeed all FcγR, bind human IgG1 and IgG3 but there are significant differences in the interaction with IgG2 and IgG4 (**Table 3**). The allelic His^{131} form of human FcγRIIA is the only receptor which avidly binds human IgG2 complexes, while FcγRIIA-Arg^{131} binds IgG2 poorly (**Table 3**). However, it is possible that under circumstances of high local concentrations of opsonizing antibodies that binding interactions occur with FcγRIIA-Arg^{131} though whether there is a functional outcome is unknown (6, 111).

In contrast, FcγRIIB binds IgG4 but not IgG2 and moreover, binds IgG1 and IgG3 an approximately 10-fold lower affinity than the activating FcγRIIA. This is consistent with its powerful physiological inhibitory function as IgG binding affinities equal to or higher than the activating receptors might otherwise prevent pro-inflammatory responses that are necessary in resisting infection. Not surprisingly, FcγRIIC has the same IgG binding properties at FcγRIIB (6).

Other factors that affect interactions between IgG and the FcγRII are the size of the IgG immune complex (112), the distribution of epitopes (111, 113), the geometry of the Fc in the complex, and receptor localization in membrane domains (114) which may also influence the avidity of immune complex binding. The state of the cell expressing the receptor (115) can also influence interaction with IgG. FcγRIIA function may be modified by "inside-out signaling" whereby external stimuli such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and IL-3 in eosinophils (116) and N-formylmethionyl-leucyl-phenylalanine (fLMP) in neutrophils increase receptor avidity (117). The mechanism for this FcγRIIA "activation" is unknown but could involve receptor dimer forms (5, 115, 117, 118). This inside-out signaling has also been identified for the high affinity IgG receptor, FcγRI,

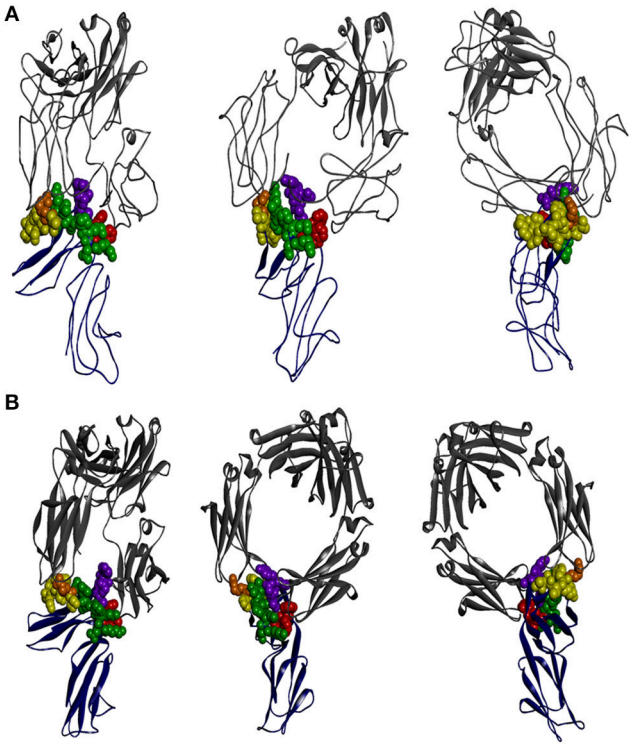

FIGURE 2 | The interaction of IgG-Fc with FcγRIIA and FcγRIIB is similar. The perspectives shown are of two ectodomains of the **(A)** FcγRIIA [adapted from 3RY6 (61)] and **(B)** FcγRIIB [adapted from 3WJJ (123)] (shown in dark blue) in complex with IgG-Fc (shown in gray). The structural components of the receptor contributing to IgG binding are the two tryptophan residues that form the Trp sandwich (red), the BC loop (green), the C′E strand (yellow), and the FG loop (purple), with the "high/low resonder" polymorphic residue His^{131}Arg highlighted (orange).

where it is associated with cytoskeletal-dependent clustering of receptors (119).

X-ray crystallographic structural data is available for all FcγR but only in complex with the native or mutated IgG1 (61, 120–122). It is clear that the interaction of FcγRIIA and FcγRIIB with IgG1 is asymmetric. The "bent" FcγR extracellular region of one FcγR molecule inserting between, and making contacts with, both IgG1 H-chain Fcs, as is also the case with other FcγR (**Figure 2**) (2, 124). The key conclusion from these studies is that the principal contact regions of the FcγRIIA and FcγRIIB are similar and occur predominantly within the second domain BC loop, C strand, C′E loop, and the FG loop, with a contribution of the interdomain linker. The BC loop and the interdomain linker provide the two critical tryptophan residues, conserved in all FcγR, that sandwich the Pro^{331} of the IgG1 CH2 FG loop.

The lower hinge of IgG has a dominant role in determining the specificity of FcγR interactions. In the case of IgG1, the lower hinge residues, $Pro^{233}Leu^{234}Leu^{235}Gly^{236}Gly^{237}$, of both H-chains form extensive contacts with FcγRIIA (61). Interestingly, this region is quite different in IgG2 (Pro,Val,Ala,Gly) and suggests that the IgG2 interaction with FcγRIIA may be quite distinct at the atomic level but as yet no structure of IgG2 in complex with FcγRIIA is known. Nonetheless, the IgG1:FcγRIIA complex structure suggests that the preferential IgG2 binding by FcγRIIA-H^{131} over FcγRIIA-R^{131}–the "high/low responder" polymorphism (125)—may be explained structurally by the smaller histidine side chain more readily accommodating interaction with the Fc adjacent to the lower hinge compared to the longer arginine side chain (61).

The structural basis for the effect of the rare $Gln^{127}Lys$ polymorphism that also affects IgG2 binding is interesting (126). The Lys^{127} does not appear to make contact with the IgG1 Fc and sits adjacent to the binding region, so that the effect on Fc binding is presumably indirect. This indicates a possible selective pressure for IgG2 binding by this receptor (126).

ROLES OF FcγRII IN HEALTH AND DISEASE

The balance between activation and inhibitory signaling is important in the control of healthy antibody dependant responses and disturbance to this balance can have adverse, but in some cases positive, consequences to health.

Genetic polymorphism studies of human *FCGR2* genes have helped to establish roles of FcγRII proteins in several autoimmune diseases and in resistance or susceptibility to infectious diseases (**Table 4**). *In vivo* mechanistic studies in experimental animal models, including transgenic and gene replacement systems, have also been helpful in establishing specific protective or deleterious roles of FcγRII in infectious disease, inflammation, autoimmunity, and cancer and have been reviewed extensively elsewhere (139–143).

Infection

The *in vivo* roles of the FcγRII receptor family in humans have been derived by extrapolation of animal studies and by genetic studies of human populations. The FcγRIIA high/low-responder polymorphism influences susceptibility to infections, as FcγRIIA-Arg^{131} has poor IgG2 binding (144, 145). Individuals expressing FcγRIIA-His^{131} are more resistant to infection by *Streptococcus pneumonia*, *Haemophilus influenza,* and *Neisseria meningitides*. This is potentially due to more avid binding of IgG2 by FcγRIIA-His^{131} over FcγRIIA-Arg^{131}, consequently resulting in more efficient effector responses such as uptake by phagocytes, induction of degranulation and elastase release by granulocytes *in vivo* (144, 146, 147).

FcγRs do not function in isolation under physiological conditions *in vivo* and it is notable that co-operation between Toll-like receptors (TLRs) and FcγRs is an important feature of effective pathogen elimination (148). TLRs are often co-expressed with FcγRIIA and co-engagement results in enhanced functional responses of these individual receptors, e.g., enhanced TNFα, IL-23, and IL-1β release by DCs (35, 149, 150).

The role of FcγR in HIV is complex and apparently conflicting data may reflect different aspects of HIV infection and clinical outcomes. In a small study of immunocompetent patients who had undergone successful and early antiretroviral treatment, who expressed FcγRIIA-His^{131}, and had a IgG2 response

TABLE 4 | Function or clinical association of polymorphic residues of FcγRII.

Receptor	Polymorphism	Function/clinical association	Reference
FcγRIIA	Gln27Trp (rs9427397, rs9427398)	Impaired calcium mobilization and MAP kinase phosphorylation; associated with CVID	(127)
	Gln127Lys	Gln^{127} interferes with the interaction of adjacent receptor residues with IgG2	(126)
	His131Arg (rs1801274)	His^{131} able to bind IgG2; both forms associated with autoimmune disease; allograft rejection and mAb cancer treatment outcomes	(128, 129)
	c.7421871A>G	Permits alternative splicing of the C1* exon resulting in expression of "hyperactive" FcγRIIA3. Risk factor for IVIg anaphylaxis.	(11, 12)
	Hypomethylation	Increased susceptibility genes for Kawasaki disease and IVIg resistance	(130)
FcγRIIB	Promoter haplotype (rs3219018, rs34701572)	Deregulated FcγRIIB expression may contribute to pathogenesis	(59, 131)
	Ile232Thr (rs1050501)	Thr^{232} allele does not partition to lipid rafts and is associated with impaired regulation of ITAM signaling, predisposing to SLE but protective for malaria	(132–137)
	Tyr235Asp	Asp^{235} has reduced binding, internalization and signaling	(95, 96)
FcγRIIC	Gln13stop	Commonly referred to as the ORF/Stop polymorphism, determines functional expression of receptor, may contribute to autoimmune disease	(105, 106)
	Gln57stop (rs1801274)	Unknown mechanism, associated with autoimmune disease and vaccine efficacy for HIV	(106, 138)

to a gp120 vaccine regime, there was a partial control of viral replication during interruption of anti-retroviral therapy (151). However, analysis of the Vax004 gp120 vaccine trial found no evidence of association of FcγRIIA polymorphism with protection against HIV infection, although this was an unsuccessful vaccine trial overall (152). HIV studies have emphasized the protective role of NK cell FcγRIIIA in antibody dependent cellular cytotoxicity. However, recent studies have found a potent role for FcγRIIA in the protective functions of macrophages and neutrophils, which are abundant effectors at the mucosal sites of HIV acquisition (153). HIV co-infections generate an even more complex clinical picture. FcγRIIA-His^{131} homozygous individuals are more susceptible to developing AIDs-related pneumonia, and have an increased risk of placental malaria in HIV-infected women (154) and other perinatal infections (155, 156).

While few resting CD4 T cells express FcγRIIA, these cells are highly relevant to HIV research. Resting CD4 T cells latently infected with HIV are an important target in strategies to eliminate HIV in anti-retroviral therapy (ART) patients, as these quiescent cells provide safe harbor for "silent" virus that, upon reactivation, causes viral recrudescence within weeks of treatment interruption. FcγRIIA was reported as a surface marker of this key quiescent population in ART patients (157) but other studies found no enrichment of HIV proviral DNA by sorting CD4 T cells based on FcγRIIA expression (52, 158). Rather than on resting CD4 T cells, FcγRII expression was mostly on activated CD4 cells associated with transcriptionally active virus (159). Furthermore, another study sorted a $CD4^+$ population that apparently expressed FcγRIIB, not FcγRIIA. However, these FcγRIIB$^+$ cells derived from contaminating B cells, occurring as T-B cell doublets, and also from single CD4 T cells, with a punctate staining pattern that included other B cell markers, and was suggestive of trogocytosis rather than intrinsic CD4 T cell expression (52). These studies indicate some of the technical challenges that can accompany determining FcγR expression.

Though the numbers are small there is suggestive evidence that polymorphism in the *FCGR2C* locus, in particular *FCGR2C*-126 C>T SNP was associated with a protective anti-HIV vaccination response. In the RV144 vaccine trial, individuals homozygous for *FCGR2C*-126C/C had an estimated vaccine efficacy of 15% whereas individuals homozygous for the *FCGR2C*-126T/T or heterozygous−126 C/T had an estimated vaccine efficacy of 91% (138). Whether this association relates to effector function via a functional FcγRIIC protein or is due to linkage to another effector system encoded in this chromosomal region is uncertain (109).

FcγRs also have an established role in antibody-dependent enhancement (ADE) of dengue virus (DENV) infection. Immune complexes of DENV opsonized with non- or sub-neutralizing levels of antibodies interact with FcγRs on monocytes, macrophages, and dendritic cells, led to increased uptake, viral replication, and more severe infection (160). In keeping with its modulating role, FcγRIIB inhibits ADE in experimental systems (161). Indeed, while FcγRIIA facilitates DENV entry, mutation of the ITAM to an ITIM significantly inhibited ADE, and conversely, replacing the inhibitory motif in FcγRIIB with an ITAM, conferred ADE capacity (162).

The hypo-functional FcγRIIB-Thr^{232} variant is enriched in populations from malaria endemic areas. This suggests that reduced FcγRIIB modulation of responses and a consequential enhancement of B cell and inflammatory cell activation confers a survival advantage in these populations (132, 163). Indeed, enhanced activatory FcR responses including increased phagocytic capacity and TNF production by innate cells and enhanced B cell responses is evident by elevated malaria specific antibody titers (164).

Interestingly, the FcγRIIB-Thr^{232} polymorphism has been shown to confer increased phagocytosis of antibody opsonized

bacteria by monocyte-derived macrophages (132). Models suggest FcγRIIB is integral for the balance between efficient pathogen clearance and the prevention of the cytokine-mediated effects of sepsis (163). In geographic areas where there is less infectious disease pressure, FcγRIIB-Thr^{232} is associated with susceptibility to autoimmunity.

FcγR in Autoimmunity

Imbalance between inhibitory and activatory FcγR functions predisposes individuals to pro-inflammatory autoimmune disease. FcγRIIA activation induces the production of pro-inflammatory cytokines, including IFN and TNFα, which are active in the promotion of inflammation, systemic lupus erythematosus (SLE), Kawasaki disease (KD), Grave's disease, and Rheumatoid Arthritis (RA) (35, 165–167).

The FcγRIIA-His^{131} allelic form is associated with other autoimmune diseases, including Guillain-Barré syndrome, ulcerative colitis and KD, possibly due to increased inflammatory cell activation via IgG2 (168–170).The FcγRIIA-Arg^{131} allelic form is associated with susceptibility to SLE, angina pectoris, acute coronary syndrome (ACS), myasthenia gravis, and RA (171–174). This may be related to the impaired ability of FcγRIIA-Arg^{131} to process and recycle IgG2, causing the release of pro-inflammatory cytokines, aggravating disease (175, 176).

Other FcγRIIA polymorphisms, although less well characterized, are associated with inflammatory diseases. Recently a glutamine/tryptophan polymorphism at position 27 (Gln^{27}Trp) has been identified, where homozygous individuals were over represented in CVID (127). No difference in expression was observed and FcγRIIA-Trp^{27} had modest impairment of calcium mobilization and MAP kinase phosphorylation *in vitro* (127).

Epigenetic modifications of *FCGR2A* such as hypomethylation have also been described in CVID patients, particularly at the promoter CpG site cg24422489 (130, 169). This increased susceptibility for KD and resistance to Ig replacement therapy, with significant hypomethylation of FcγRIIA in patients with acute KD and coronary artery lesions (130, 169, 177).

The recently described rare intronic A>G SNP that controls expression of the splice variant FcγRIIA3 occurs in <1% of healthy subjects (11, 12). However, it is associated with KD, immune thrombocytopenia (ITP), and CVID (11). Furthermore, severe adverse reactions in response to immunoglobulin replacement therapy occurred in patients expressing FcγRIIA3 and neutrophil activation (mediator and elastase release) was enhanced. Increased signaling by FcγRIIA3 was due to its altered membrane localization and longer membrane retention time (11, 12). Thus, increased inflammatory responses toward therapeutic IgG may paradoxically diminish the utility of the major treatment regime in this subset of CVID patients.

Polymorphism and CNV of activatory FcγRIIC is associated with increased severity of RA and ITP (106, 178). This has been attributed to expression variance in these individuals causing an imbalance between activatory and inhibitory signals.

Since the inhibitory FcγRIIB forms modulate the activation of B cells and innate effector cells, decreased expression of the FcγRIIB leads to dysregulated antibody function and increased antibody-dependant inflammatory cell responses and thus increased susceptibility to autoimmune diseases. Polymorphisms in the *FCGR2B* promoter or transmembrane domain of FcγRIIB influence receptor expression and signaling potency and are associated with susceptibility to autoimmune diseases including SLE, Goodpasture's disease, ITP, and RA (133–135, 156, 179, 180). Multiple polymorphisms in the promoter region of *FCGR2B* have been identified. The promoter haplotype *FCGR2B*−386G>C SNP in combination with *FCGR2B*-120T>A SNP (*FCGR2B*-386C +−120A) enhances promoter activity and transcription, however this enhanced haplotype has low prevalence (59, 131). *FCGR2B*-343G>C SNP is enriched in European American SLE patients and homozygous expression of *FCGR2B*-343C is linked to SLE susceptibility (131, 179). This is due to decreased AP1 transcription complex binding, which causes decreased FcγRIIB expression on B cells and macrophages and altered antigen clearance (179).

The frequency of the transmembrane polymorphism FcγRIIB-Thr^{232}Ile differs among different ethnic populations, with FcγRIIB-Thr^{232} associated with SLE in Asian but not African American or European populations (134). FcγRIIB-Thr^{232} shows reduced lateral mobility in the membrane which impairs its ability to inhibit the co-localization of BCR and CD19 microclusters and consequent B cell activation (181). This causes increased B cell and myeloid cell activation (133, 136, 137), which elevates B cell (antibody) responses and heightens IgG-dependant pro-inflammatory responses, resulting in autoimmunity.

Cancer

The roles of FcγR in cancer relate largely to the harnessing of antibody-dependant effector functions such as ADCC or ADCP by therapeutic mAbs during the treatment [reviewed in (2, 139)]. However, it also appears that mAb therapy may also have long term therapeutic benefits. Studies on DCs indicate that FcγRIIA activation is necessary and sufficient to induce a strong T cell anti-tumor cellular immunity inducing long term anti-tumor vaccine-like or "vaccinal effects" in humanized mice (48). Engagement of FcγRIIA induced DC maturation and up-regulation of costimulatory molecules, priming them for optimal antigen presentation and cross-presentation, thus stimulating long-term anti-tumor T cell memory (48).

Conversely, the inhibitory role of FcγRIIB may be disadvantageous to antibody-based therapies and other immune stimulating therapies. Thus, blocking inhibitory function of FcγRIIB on effector cells or antigen presenting cells such as DCs might be a strategy to enhance anti-tumor immune responses during immunotherapy (18, 182, 183).

HARNESSING OR TARGETING FcγRII FOR ANTIBODY BASED THERAPIES

Monoclonal antibodies are a versatile class of biotherapeutic drugs because of the multifunctional nature of the antibody molecule. IgG-based therapeutic mAbs are effective for the treatment of a variety of diseases due to their high specificity

and affinity for their target antigen and, in some cases, their strong induction of FcγR effector functions. Depending on the nature of the disease and molecule, the mAb efficacy may depend on one or more mechanisms of action, ranging from simple antigen neutralization, complement-dependent cytotoxicity, FcγR-dependant cellular effector functions, or inhibition via FcγRIIB. Thus, effective patient responses can be dependent on FcγR based mechanisms, e.g., altered binding due to the FcγRIIA-His^{131}Arg polymorphism, which influence the efficacy of therapeutic mAbs such as rituximab and cetuximab (184, 185).

The efficacy of the anti-EGFR mAb, cetuximab, and subsequent progression free survival was associated with expression of the His^{131} variant of FcγRIIA (185). Patients with the FcγRIIA-His^{131} genotype also responded better to rituximab treatment in non-Hodgkin's lymphoma (184). Conversely, FcγRIIB expression on lymphoma cells is a risk factor for anti-CD20 rituximab therapy failure due to FcγRIIB internalizing the CD20:rituximab complex and thereby reducing exposure of the opsonized lymphoma cell to the immune effector systems (186).

Inhibition of activatory FcγR could block early development of inflammatory disease. This has been explored experimentally in humanized mouse models of RA, using antibody fragments (or small molecules) designed to bind human FcγRIIA to inhibit disease (29, 187). Synthetic FcR mimetics have also been used to block the function of FcγRIIA *in vitro* (188) and the modulation of FcγRIIA and FcγRIIB function in humans (189).

FcγRIIB is a powerful modulator of ITAM-dependent receptors such as the BCR or high affinity FcεRI. Strategies to harness this powerful inhibitory capacity are being developed by engineering mAb Fc regions with enhanced and/or selective engagement with FcγRIIB. Such strategies rely on the co-engagement of FcγRIIB with the mAb-targeted activating receptor. This engineering of therapeutic mAbs with increased affinity to FcγRIIB has diverse clinical applications. Indeed, anti-CD19 binds the BCR complex and the engineered Fc co-engages FcγRIIB with increased affinity, suppressing B cell activation without B cell depletion (190, 191). This novel approach to treat autoimmune disease demonstrates the importance of understanding FcγR biology and interactions with IgG in order to optimally exploit antibody functions for specific therapies.

Another example is the anti-IgE, omalizumab, an effective treatment for allergic asthma by neutralizing IgE binding to FcεRI. Mutations introduced in XmAb7195, an omalizumab "equivalent" antibody, enhanced affinity for FcγRIIB. Like omalizumab, XmAb7195 binds to and neutralizes circulating IgE (71). However, its enhanced Fc interaction with FcγRIIB may also promote co-aggregation of FcγRIIB with the BCR of IgE+ B cells, and may suppress activation of the BCR, diminishing allergic antibody production. In addition, data from mouse studies suggest that the XmAb7195:IgE complexes are rapidly removed from the circulation via FcγRIIB expressed in the liver endothelium (71).

FcγR Targeted Therapies

In some autoimmune diseases, auto-antibodies activate inflammatory cell effector functions against self-antigens leading to tissue destruction. One strategy used to ameliorate this destructive pathogenesis is the use of soluble FcγRs, which compete for auto-antibody binding with cell-based FcγRs thereby preventing induction of the cell-based effector functions (1, 9). Pre-clinical studies have demonstrated that the use of these soluble FcγRs suppresses the Arthus reaction, collagen-induced arthritis, and SLE (192, 193). A soluble recombinant form of FcγRIIB, named SM101, is a potential treatment for the treatment of ITP and SLE and has progressed into clinical trials (194, 195).

Small chemical entities (SCEs) specific for FcγRIIA have also been reported to inhibit immune complex-induced responses including platelet activation and aggregation, and TNF secretion by macrophages *in vitro* (187). Furthermore, *in vivo* testing of these SCEs in FcγRIIA transgenic mice also inhibited the development and stopped the progression of collagen-induced arthritis (CIA) (187). Hence, these SCE FcγRIIA antagonists demonstrated their potential as anti-inflammatory agents for pro-inflammatory immune complex-dependent autoimmune diseases.

CONCLUSIONS

FcγRII receptors and their variants play important roles in the healthy immune response to infection, as well as in the pathologies of autoimmunity and the efficacy of therapeutic mAb treatments in cancer. Our expanding knowledge of these widely expressed FcγR and their signaling pathways may provide insight as to how we can exploit this intricate immunomodulatory system for therapeutic and diagnostic purposes. Harnessing FcR-dependent cellular effector systems through therapeutic mAbs, or by blocking effector functions, is becoming an increasingly useful tool to treat an extensive range of diseases.

AUTHOR CONTRIBUTIONS

JA drafted the manuscript. AC, BW, and PMH provided additional text and all authors reviewed the manuscript.

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Extensive Ethnic Variation and Linkage Disequilibrium at the *FCGR2/3* Locus: Different Genetic Associations Revealed in Kawasaki Disease

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The human Fc-gamma receptors (FcγRs) link adaptive and innate immunity by binding immunoglobulin G (IgG). All human low-affinity FcγRs are encoded by the *FCGR2/3* locus containing functional single nucleotide polymorphisms (SNPs) and gene copy number variants. This locus is notoriously difficult to genotype and high-throughput methods commonly used focus on only a few SNPs. We performed multiplex ligation-dependent probe amplification for all relevant genetic variations at the *FCGR2/3* locus in >4,000 individuals to define linkage disequilibrium (LD) and allele frequencies in different populations. Strong LD and extensive ethnic variation in allele frequencies was found across the locus. LD was strongest for the *FCGR2C*-ORF haplotype (rs759550223+rs76277413), which leads to expression of FcγRIIc. In Europeans, the *FCGR2C*-ORF haplotype showed strong LD with, among others, rs201218628 (*FCGR2A*-Q27W, $r^2 = 0.63$). LD between these two variants was weaker ($r^2 = 0.17$) in Africans, whereas the *FCGR2C*-ORF haplotype was nearly absent in Asians (minor allele frequency <0.005%). The *FCGR2C*-ORF haplotype and rs1801274 (*FCGR2A*-H131R) were in weak LD ($r^2 = 0.08$) in Europeans. We evaluated the importance of ethnic

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variation and LD in Kawasaki Disease (KD), an acute vasculitis in children with increased incidence in Asians. An association of rs1801274 with KD was previously shown in ethnically diverse genome-wide association studies. Now, we show in 1,028 European KD patients that the *FCGR2C*-ORF haplotype, although nearly absent in Asians, was more strongly associated with susceptibility to KD than rs1801274 in Europeans. Our data illustrate the importance of interpreting findings of association studies concerning the *FCGR2/3* locus with knowledge of LD and ethnic variation.

Keywords: Fc-gamma receptor, FCGR polymorphism, linkage disequilibrium, Kawasaki disease (KD), immunogenetics

INTRODUCTION

The human cellular receptors for Immunoglobulin G (IgG), the Fc-gamma receptors (FcγR), have an important role in immunity by linking the adaptive and innate immune systems. Many genetic variations in the genes encoding FcγRs have been found to be associated with auto-immune (1–5), auto-inflammatory (6–8), and infectious diseases (9, 10), and with efficacy of immunotherapy in cancer patients (11–15). Several activating and one single inhibitory FcγR (FcγRIIb) exist, with differential expression on various leukocyte subsets (16, 17). Human FcγRs can be distinguished into one high-affinity receptor (FcγRI) and five low-affinity FcγRs (the different isoforms of FcγRII and FcγRIII) (16, 17). All five genes encoding the low-affinity FcγRs (*FCGR2A, FCGR2B, FCGR2C, FCGR3A*, and *FCGR3B)* are located in a complex gene cluster at chromosome 1q23.3. Many functionally relevant single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) are described in the *FCGR2/3* locus, leading to altered receptor functions ranging from different binding affinity to IgG to complete absence of expression of certain genes (17–19). The *FCGR2/3* locus involves a segmental duplication, making it constitutively difficult to genotype because of the high degree of homology between the genes (18, 20). Due to the close proximity of all the five different *FCGR2* and *FCGR3* genes, the polymorphic variants in these genes are likely to be in strong Linkage Disequilibrium (LD). However, except for some incidental reports on LD between some of the SNPs (21–24), a comprehensive analysis of LD between the functional variants at this locus has not been previously performed.

One of the diseases in which only one genetic variant of the *FCGR2/3* locus has been thoroughly studied is Kawasaki Disease (KD). KD is an acute systemic vasculitis that predominantly occurs in children <5 years (25). About 25% of untreated KD patients develop coronary artery aneurysms, which may lead to ischemic heart disease, myocardial infarction and sudden death at young age (26). Although the etiology of KD remains unknown, the general consensus is that KD reflects an abnormal inflammatory response to an unknown infectious trigger in genetically susceptible individuals. Standard treatment consists of a single infusion of high-dose intravenous immunoglobulins (IVIg) in combination with aspirin (27). Although the mechanism of action of IVIg in KD is unclear, early treatment shortens the duration of fever and reduces the incidence of coronary artery aneurysms to less than 5% (28). Since IVIg therapy is effective in the majority of patients, the receptors for IgG, the Fc-gamma Receptors (FcγRs), are of particular interest in KD research.

In our GWAS study on KD (6), we identified the *FCGR2A*-131H SNP (rs1801274) to be associated at genome-wide significance. This variant results in a substantial difference in the ability of FcγRIIa to bind the human IgG2 subclass (19). rs1801274 shows the strongest evidence of association with KD and this finding has been intensively studied and validated in a number of cohorts of varying ethnicity (6, 7, 29–34). Apart from the *FCGR2A*-H131R SNP (rs1801274), only a few other SNPs in this locus have been evaluated for KD susceptibility, without any significant association (29–31). Nevertheless, because of the sequence homology and the genetic complexity, a very large part of the *FCGR2/3* locus was not covered in GWAS or other studies before. Hence, we postulated that other variants at the locus may also play a role in KD susceptibility, which could either be tagged by *FCGR2A*-131H (rs1801274), or act independently. To address this, we performed further fine-mapping of the *FCGR2/3* gene cluster in a case-control as well as a family-based linkage study with a total of 1,028 patients with KD, and genotyped healthy control individuals of different ethnic groups to define LD and ethnic variation. We used a previously developed accurate multiplex ligation-dependent probe amplification (MLPA) assay covering all the functionally relevant SNPs and CNVs at the *FCGR2/3* locus (5).

In the present study, including more than 4,000 individuals, we found marked ethnic differences in allele frequencies for most of the SNPs and CNVs. The most prominent difference was observed for the *FCGR2C*-ORF haplotype, which we have previously shown to result in expression of the activating FcγRIIc (35). In most individuals, FcγRIIc cannot be expressed as a result of a polymorphic stop codon in exon3 (rs759550223), but the expressed *FCGR2C*-ORF haplotype is associated with susceptibility to immune thrombocytopenic purpura (5). We now show that the *FCGR2C*-ORF haplotype is virtually absent in Asian and African populations. *FCGR2C*-ORF is in very strong LD with several other SNPs in the European population, but could be identified as a novel susceptibility haplotype for KD in this population, independent of the *FCGR2A*-H131R SNP. Our comprehensive analysis of the *FCGR2/3* locus will greatly contribute to a better understanding of the relevance of the different FcγRs in inflammatory diseases.

SUBJECTS AND METHODS

Study Populations

KD Cases

Unrelated KD cases were recruited from Australia, The Netherlands and the United States. All cases from Australia (109) and the United States (62) were also included in our previous GWAS (6), whereas the cases from the Netherlands (234) consisted of 166 cases from the GWAS and 68 new cases. There was no overlap with patients in the study previously reported by Biezeveld et al (30). The diagnosis of KD was based on the standard diagnostic clinical criteria from the American Heart Association.

Cohorts of Control Subjects

Europeans

Since no DNA of the control population in our previous GWAS was available, we genotyped a new group of unrelated controls of European descent, consisting of healthy individuals from Austria (478), Australia (156), The Netherlands (199), and the United Kingdom (86). All were of European descent by self-reported ethnicity (36, 37).

Chinese

The Chinese population consisted of 428 healthy individuals from Canada of Han-Chinese descent, all of which were grandparent-proven Han-Chinese.

African

The South African population consisted of 149 healthy blood donors of African descent by self-reported ethnicity as reported before (38). The Ethiopian population consisted of 142 healthy blood donors of African Ethiopian descent by self-reported ethnicity (38). The West African population consisted of 65 sickle-cell disease patients from the Netherlands, all of which were of West-African descent by self-reported ethnicity, including individuals from Ghana (52), Nigeria (4), Sierra Leone (4), Togo (3), and Cameroon (2). The Surinam population consisted of 78 sickle-cell disease patients of African Surinamese descent by self-reported ethnicity. The Antillean population consisted of 6 sickle-cell disease patients from the Netherlands who were from Curaçao and were of African Caribbean descent by self-reported ethnicity, and 68 healthy blood donors from Curaçao who were of African Caribbean descent by self-reported ethnicity as described previously (38).

Family-based association study

623 KD patients (none overlapping with the case control study) were included, consisting of KD patients from the United States (386, of which 348 complete trios and 38 incomplete trios, 153 European), Australia (104, all complete trios, 72 European) and the Netherlands (98, all complete trios, 82 European) and Italy (35, all complete trios, all Mediterranean). All KD patients in the family-based association study from the United States and Australia were included in our previous GWAS (6), the patients from the Netherlands and Italy were new.

In total, 4,091 individuals were genotyped. **Table S1** provides an overview of all individuals. This study was carried out in accordance with the recommendations of the Kawasaki Study Protocol approved by the Medical Ethical Committee at the Academic Medical Centre in Amsterdam, the Netherlands, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Medical Ethical Committee at the Academic Medical Centre in Amsterdam, the Netherlands and by the medical ethical committees of the other participating centers.

Clinical Data

Clinical information was collected by review of the clinical KD registries. CAAs were defined based on the definition of the Japanese Ministry of Health or Z-scores >2.5 according to the Boston Z-score data. According to the definition of the Japanese Ministry of Health a coronary artery was considered abnormal if the diameter of the internal lumen was > in children younger than 5 years or > in a child aged 5 years or older, or if the internal diameter of a segment was at least 1.5 times larger than that of an adjacent segment. IVIg response was determined in the patients receiving treatment with IVIg within 11 days after the disease onset. Patients who received more than one dose of IVIg because of persistent or recrudescent fever more than 36 h after the initial IVIg dose were defined as IVIg non-responders.

Genotyping by MLPA and Construction of Haplotypes From MLPA Data

The MLPA assay was performed according to the manufacturer's protocol, essentially as described previously (5, 39) and is described in great detail in the **Supplemental Methods**.

Flow Cytometry, Gene Expression Microarray and RT-qPCR

Flow cytometry, gene expression microarray and RT-qPCR were performed as described in the **Supplemental Methods**.

Statistical Analysis

Genotype/Allele Frequencies and Linkage Disequilibrium

Differences in copy number and allele frequencies between (sub)populations and differences in allele frequencies between groups of individuals with normal, decreased and increased copy number were tested using Fisher's Exact test. Haplotype frequencies and linkage disequilibrium (expressed as r^2 or D') between (multiallelic) markers were estimated in the populations and the parents from the KD trios using the gap package (40) (version 1.1-12).

Association With Susceptibility to Kawasaki Disease (KD)

In the case-control study, genotype frequencies were compared between KD cases and healthy controls using Fisher's exact test and odds ratios were estimated using (multiple) logistic regression. In the parent-affected offspring trios, the association between KD and the markers was examined using the (multimarker) FBAT (TDT) test statistic from the FBAT toolkit

(41). Results from the case-control and KD trios were meta-analyzed using a fixed effect model and the generic inverse variance method following an approach described by Kazeem and Farrall (42) and using Review Manager software (Version 5, Cochrane Collaboration).

Comparison of Expression Levels

In case of multiple expression values per donor, the mean of these values was taken for the statistical analyses. Expressions between groups were compared using Mann-Whitney tests (two groups) or a Kruskal-Wallis test with *post-hoc* Mann-Whitney tests (>2 groups) using GraphPad Prism 6.02.

Apart from the TDT and meta-analyses and the expression analysis, all statistical analyses were carried out using R software (Version 3.0.3, R Core Team). A p-value below 0.05 was considered as statistically significant.

RESULTS

Characterization of the *FCGR2/3* Locus

The *FCGR2/3* locus is a complex region due to the presence of a large segmental duplication and copy number variants (CNV) (18, 43). MLPA was previously shown to accurately call copy number variation at the *FCGR2/3* locus (5, 20). We used the MLPA to accurately identify all eight known functional SNPs and haplotypes, as well as the four CNV regions (CNRs), at the *FCGR2/3* locus, which have previously been associated with various autoimmune and infectious diseases (**Figure 1** and **Table S2**).

Allele Frequencies of CNV and SNPs at the *FCGR2/3* Locus Vary Among Different Ethnic Groups, Especially for the Classic and Nonclassic *FCGR2C*-ORF Haplotypes

The frequencies of many of the functional SNPs and CNVs have been reported to vary among different ethnic backgrounds (10, 21, 44–47), but information about the *FCGR2C* haplotypes is yet to be established. To explore differences in frequencies of SNPs and CNRs between several ethnic groups, we genotyped and compared large groups of healthy human subjects. Significant differences ($P < 0.05$) between ethnic groups were found for CNRs and for all SNPs except the *FCGR3A*-V158F SNP, which had no difference in frequency among all groups (**Table 1**). Analysis of subgroups within the European and African populations revealed subtle differences within the European population and marked differences within the African population (**Table S3**).

Among the groups included, the largest difference in allele frequency was revealed for the *FCGR2C*-haplotypes. *FCGR2C* consists of three haplotypes; the *FCGR2C*-Stop pseudogene that is not expressed as a result of the *FCGR2C*-Q57X SNP (rs759550223), its expressed counterpart, the so-called classic *FCGR2C*-ORF with an open reading frame at rs759550223, and the nonclassic *FCGR2C*-ORF, which has an open reading frame at rs759550223 but has an almost complete lack of expression as a result of a splice site mutation in intron7 (rs76277413) (35). **Figure 1E** gives a schematic overview of the haplotypes of *FCGR2C*. The classic *FCGR2C*-ORF haplotype results in the expression of FcγRIIc as an activating IgG receptor on myeloid cells and NK cells, as we have characterized previously (5, 48). We now formally demonstrate that the nonclassic *FCGR2C*-ORF haplotype can be determined by MLPA (see **Supplemental Methods** and **Table S4** for a description), as expression of FcγRIIc is indeed low to absent in individuals genotyped as nonclassic *FCGR2C*-ORF by MLPA (**Figure 2**, gating strategy **Figure S1**). The slight difference in staining levels compared to individuals with the *FCGR2C*-stop variant shows that there is some residual expression of FcγRIIc protein, but this is less than 10% of the expression in classic *FCGR2C*-ORF individuals. These haplotypes were markedly different among different ethnic groups; the classic *FCGR2C*-ORF haplotype was virtually absent in Chinese (present in 2 out of 428 individuals, minor allele frequency <0.005%) and rare in the different African populations, whereas the nonclassic *FCGR2C*-ORF was more prevalent in African populations compared to Europeans (**Table 1** and **Figure 2C**).

Linkage Disequilibrium at the *FCGR2/3* Locus Defined

Because many functionally relevant SNPs in the *FCGR2/3* locus are located in close proximity to each other, the SNPs in *FCGR* genes are likely to be in strong LD, which can greatly complicate the interpretation of genetic association studies. From the control samples of the different ethnic reference populations, we first calculated the background LD pattern based on the SNPs and haplotypes in the individuals that did not show CNV (r^2 in **Figure 3**, D' in **Figure S2**).

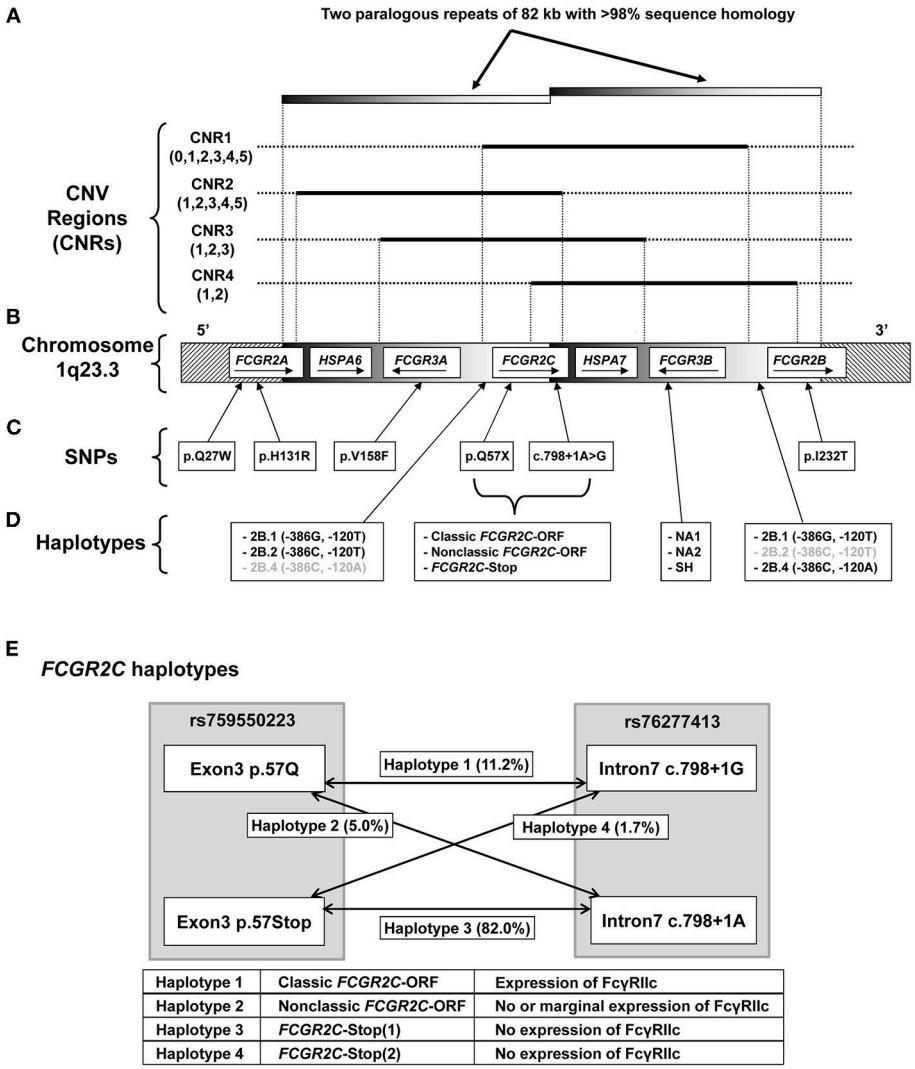
In the European population, we found strong LD of the classic *FCGR2C*-ORF haplotype (rs759550223 and rs76277413) with several of the other SNPs in the region. First, the classic *FCGR2C*-ORF haplotype was in almost complete LD ($r^2 = 0.92$) with the 2B.2 promoter in *FCGR2C* (rs149754834). Furthermore, there was strong LD between the classic *FCGR2C*-ORF variant and *FCGR2A*-27W (rs201218628, $r^2 = 0.63$) and with the 2B.4 promoter haplotype in *FCGR2B* (rs143796418, $r^2 = 0.40$). Weaker LD was observed for the classic *FCGR2C*-ORF haplotype with *FCGR3A*-158V (rs396991, $r^2 = 0.24$) and *FCGR2A*-131H (rs1801274, $r^2 = 0.08$).

In the Chinese population, LD for the classic *FCGR2C*-ORF haplotype appeared similar to the LD in Europeans, but this was based only on 2 individuals.

In the African population, LD was also found for the *FCGR2C*-ORF haplotype with several of the variants, but in general this LD was weaker than in Europeans (**Figure 3**, second panel).

The previously described LD between *FCGR3A*-158V (rs396991) and *FCGR2A*-131H (rs1801274) (21, 23) was confirmed in the European and African population, although relatively weak ($r^2 = 0.06$). We show now that this LD was reversed in the Chinese population, i.e., *FCGR3A*-158F (rs396991) and *FCGR2A*-131H (rs1801274) were in weak LD ($r^2 = 0.04$).

We then investigated LD between CNV and SNPs for all of the CNRs known at the locus. Because the standard measurements of LD (r^2 and D') cannot be calculated in areas with CNV, we performed this analysis by calculating allele frequencies



Haplotype 1	Classic *FCGR2C*-ORF	Expression of FcγRIIc
Haplotype 2	Nonclassic *FCGR2C*-ORF	No or marginal expression of FcγRIIc
Haplotype 3	*FCGR2C*-Stop(1)	No expression of FcγRIIc
Haplotype 4	*FCGR2C*-Stop(2)	No expression of FcγRIIc

FIGURE 1 | Genomic organization of the *FCGR2/3* locus. Overview of the *FCGR2/3* locus at 1q23.3. **(A)** CNV at the locus occurs always in regions containing a series of genes, termed CNV regions (CNRs) (18, 21). Numbers between brackets indicate observed copy number for that CNR in the individuals tested in this study, black lines indicate the extent of the different CNRs. Gray shaded bars indicate the extent of two paralogous repeats of the locus. The novel rare CNR4 that we recently described (18) was found in 4 of the individuals included in this study, and was combined with the similar CNR1 in this study for reasons of simplicity. **(B)** Overview of the genes in the locus with their orientation. Depiction of genes is not to scale. **(C)** Functional SNPs at the locus, indicated by single letter amino acid code and amino acid position, except for the splice variant c.798+1A>G. All these SNPs were determined in this study. **(D)** Functional haplotypes at the locus. 2B.1, 2B.2, and 2B.4 are haplotypes of two SNPs (at nucleotide positions −386 and −120 relative to the start of translation) in the otherwise identical promoter regions of *FCGR2B* and *FCGR2C* (haplotypes in gray are very rarely found in that particular gene). *FCGR3B* NA1, NA2, and SH are haplotypes determined by six SNPs. These haplotypes determine the different allotypes of Human Neutrophil Antigen 1 (HNA1) involved in neutrophil alloimmunization, and respectively encode the HNA1a/HNA1b/HNA1c antigenic variants. **(E)** Schematic representation of the different haplotypes of *FCGR2C* haplotypes, which determine expression of FcγRIIc. *FCGR2C*-Stop (1) and *FCGR2C*-Stop (2) haplotypes are similar in function and expression and are taken together as *FCGR2C*-Stop throughout the manuscript. Percentages represent allele frequencies of the different haplotypes in European healthy controls.

for groups of individuals with normal (2 copies), decreased (≤1 copies) or increased (≥3 copies) copy number of at least one CNR and analyzed significant differences by Fisher's exact test.

Results for CNR1 are shown in **Table S5**. For CNR1, strong LD was found between increased copy number and the nonclassic *FCGR2C*-ORF haplotype (rs759550223 and rs76277413), both in the European and African population. Increased copy number in CNR1 also revealed strong LD with the *FCGR3B*-SH (rs5030738) haplotype in the European, but not in the African population. Some other SNPs [*FCGR2A*-H131R (rs1801274); *FCGR3A*-V158F (rs396991); *FCGR2B*-I232T (rs1050501)] were also associated with changes in CNV in CNR1.

For the less prevalent CNR2, LD was found only for rs1050501 in the European population (All results for CNR2 are shown in **Table S6**).

For the rare CNR3, no statistically significant LD was found at all (data not shown).

TABLE 1 | Frequencies of CNVs (CNRs, proportion of individuals with that number of copies is shown) and SNPs (allele frequencies are shown).

Variant		European ($n = 919$)	Chinese ($n = 428$)	African ($n = 508$)	Fisher's exact
CNR1					
FCGR3B + *FCGR2C*	0 copies	0.00	0.00	0.00	
	1 copy	0.07	0.09	0.11	
	2 copies	0.83	0.73	0.73	
	3 copies	0.09	0.17	0.14	
	4 copies	0.01	0.01	0.01	**<0.0001**
CNR2					
FCGR3A + *FCGR2C*	1 copy	0.01	0.01	0.01	
	2 copies	0.94	0.96	0.96	
	3 copies	0.04	0.04	0.03	
	4 copies	0.00	0.00	0.00	0.87
CNR3					
FCGR3A + *FCGR2C*	1 copy	0.00*	0.00	0.00*	
	2 copies	1.00	0.98	1.00	
	3 copies	0.00	0.02	0.00	**<0.001**
FCGR2A					
	131 H	0.54	0.67	0.44	
	131 R	0.46	0.33	0.56	**<0.0001**
	27 Q	0.88	1.00	0.89	
	27 W	0.12	0.00	0.11	**<0.0001**
FCGR3A					
	158 F	0.64	0.64	0.64	
	158 V	0.36	0.36	0.36	0.94
FCGR2C					
	Stop	0.84	1.00	0.90	
	Classic ORF	0.11	0.00	0.02	
	Nonclassic ORF	0.05	0.00	0.08	**<0.0001**
Promoter haplotype	2B.1	0.89	1.00	0.95	
	2B.2	0.11	0.00	0.05	**<0.0001**
FCGR3B					
	NA1	0.35	0.62	0.38	
	NA2	0.62	0.38	0.46	
	SH	0.02	0.00	0.15	**<0.0001**
FCGR2B					
	232I	0.88	0.74	0.73	
	232T	0.12	0.26	0.27	**<0.0001**
Promoter haplotype	2B.1	0.90	1.00	0.99	
	2B.4	0.10	0.00	0.01	**<0.0001**

*Fisher's exact test: Overall P for differences between populations for that variation is shown. P-values < 0.05 are shown in bold. *1 European and 1 West African individual showed a deletion of CNR3.*

Association of SNPs and CNV at the *FCGR2/3* Locus With Susceptibility to KD

After defining the background allele frequencies and LD of the functional SNPs and CNV in the control groups, we then analyzed the full content of variants in the *FCGR2/3* locus for susceptibility to KD, now also including the SNPs and CNV in the region that had not been covered in our previous GWAS study (6). We performed a case-control study in 405 KD cases and the cohort of 919 controls described above, all of European descent. For a family-based association study, 586 complete trios and 37 incomplete trios were genotyped. The characteristics of the KD patients are shown in **Table S7**.

Case-Control Study

Genotype and allele frequencies of CNVs and SNPs are shown in **Table 2**. Several significant differences between cases and controls were observed, the most significant being the classic *FCGR2C*-ORF (rs759550223 and rs76277413) (15.7% vs. 11.2%, $P = 0.002$). Other significantly associated SNPs were the 2B.2 promoter in *FCGR2C* (rs149754834) (15.3% vs. 10.8%, $P = 0.009$), the *FCGR2A* 27Q>W SNP (rs201218628) (15.3% vs. 11.9%, $P = 0.014$) and the 2B.4 promoter in *FCGR2B* (rs143796418) (12.7% vs. 10.0%, $P = 0.047$). These four significantly associated variants are in strong LD with each other (**Figure 3**). In a multiple logistic regression analysis that included all the variants, none were independently associated, but a backward regression analysis revealed the classic *FCGR2C*-ORF as the strongest predictor of KD susceptibility (data not shown).

We did not detect significant differences for any of the CNV regions, or for the other functional SNPs. Even though we detected a slight trend among the KD patients with higher frequency of the *FCGR2A*-131H (rs1801274) risk allele in the current study, this association found previously in GWAS and meta-analysis (6, 7, 33) was not replicated in this dataset of European patients and healthy controls. A multiple logistic regression analysis of only the *FCGR2C*-ORF and *FCGR2A*-131H revealed that the association of *FCGR2C*-ORF was independent of *FCGR2A*-131H (**Table 2**).

Family-Based Study on KD

In an attempt to confirm our findings, we performed a KD family-based association study in 623 family trios in which the child was diagnosed with KD. The transmission disequilibrium test (TDT) analysis revealed a significant association ($P = 0.006$) of *FCGR2A*-131H (rs1801274) (**Table 3**). For the *FCGR2C*-ORF haplotype (rs759550223 and rs76277413) and the other SNPs or CNRs tested, there was no evidence of association (except for the rare allele with two copies of *FCCR3A* on one chromosome, of which one was 158V and the other was 158F, which had only 18 informative families) (**Table 3**). Of note, the number of informative families for *FCGR2C*-ORF was also relatively small, as a result of the low prevalence of this variant (**Table 1**). Analysis of the families enabled us to construct complete haplotypes for all parental chromosomes, which confirmed the LD pattern observed in the cohort of healthy controls, both in parents without any CNV as in parents that did show CNV (**Figure S3**).

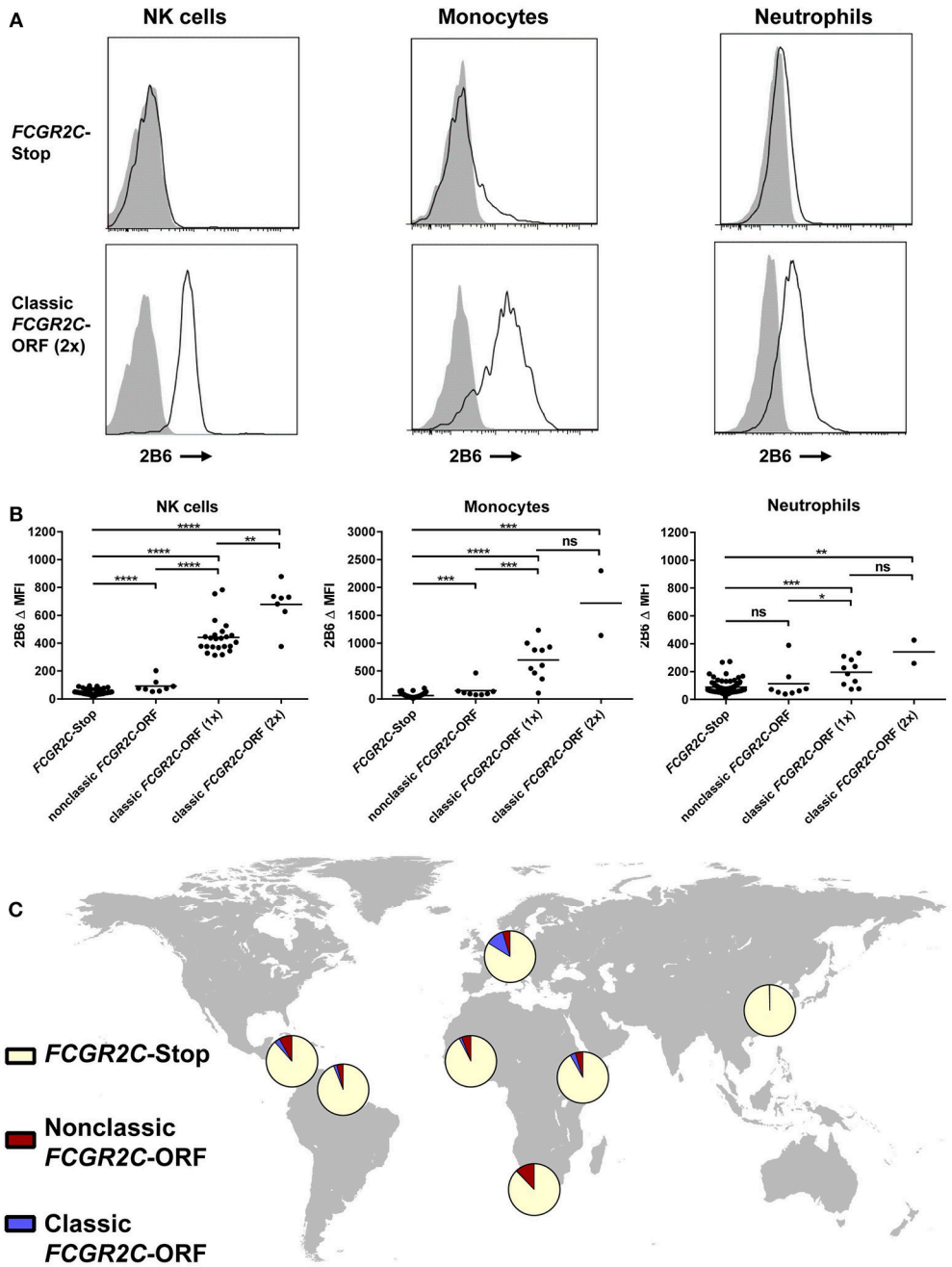


FIGURE 2 | Haplotypes of *FCGR2C* determine expression of FcγRIIc. **(A)** Representative histograms of staining with MoAb 2B6 on NK cells (left), monocytes (middle), and neutrophils (right) of an individual homozygous for *FCGR2C*-Stop variant (upper panel) and an individual homozygous for the *FCGR2C*-ORF variant (lower panel). 2B6 recognizes the extracellular domain of both FcγRIIb and FcγRIIc. In *FCGR2C*-Stop individuals, FcγRIIc cannot be expressed, therefore staining of 2B6 in those individuals must be FcγRIIb only. Black line: 2B6, gray shading: isotype control. **(B)** Summary of 2B6 staining, corrected for isotype control, on human NK cells (left), monocytes (middle) and neutrophils (right) of genotyped individuals. Y axis scale is different for monocytes than for the other cell types. Because FcγRIIb is also stained by 2B6, only cells that do not express FcγRIIb can be easily analyzed for FcγRIIc expression. Therefore, individuals with a deletion of CNR1 were excluded from the analysis of NK cells, and individuals with a 2B.4 promoter haplotype in *FCGR2B* were excluded from the analysis of monocytes and neutrophils, because these variants result in ectopic expression of FcγRIIb on NK cells (35), or myeloid cells (36), respectively. NK cell analysis: *FCGR2C*-Stop $n = 93$, nonclassic *FCGR2C*-ORF, including cases with 1 or 2 copies $n = 8$, *FCGR2C*-ORF(1x), with 1 copy of the classic *FCGR2C*-ORF haplotype, $n = 23$, *FCGR2C*-ORF(2x), with 2 copies $n = 7$. Monocyte and neutrophil analysis: *FCGR2C*-Stop $n = 99$, nonclassic *FCGR2C*-ORF, including cases with 1 or 2 copies $n = 8$, *FCGR2C*-ORF(1x), with 1 copy of the classic *FCGR2C*-ORF haplotype, $n = 10$, *FCGR2C*-ORF(2x), with 2 copies $n = 2$. Some individuals were analyzed more than once at different time points with similar results; means are shown for these. All individuals analyzed are of European descent except for five *FCGR2C*-Stop and two nonclassic *FCGR2C*-ORF individuals who were of African origin. **(C)** World map showing allele frequencies of *FCGR2C* haplotypes for different ethnic groups. MFI, median fluorescence intensity; ns, non-significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ as determined by Mann Whitney test.

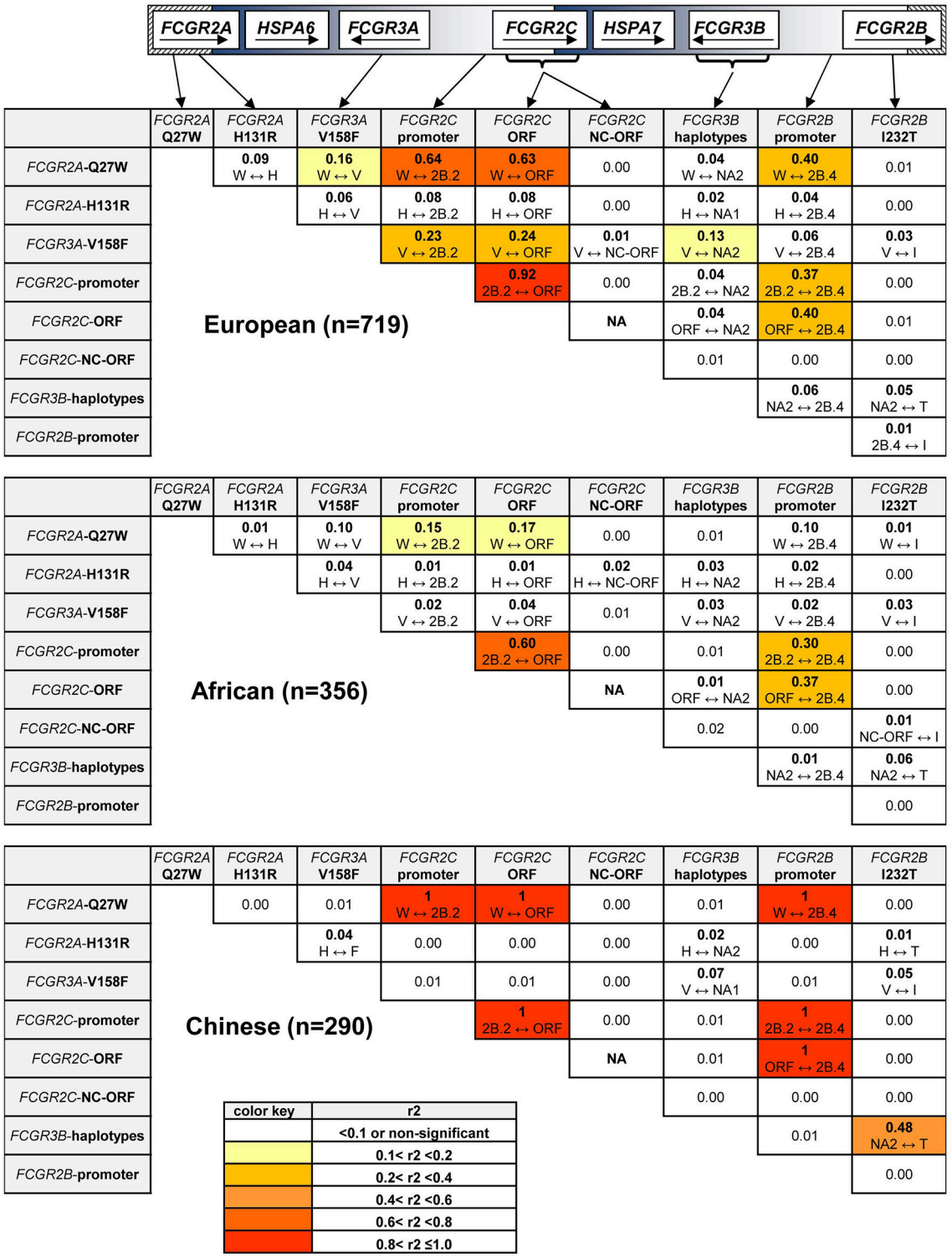


FIGURE 3 | Linkage Disequilibrium at the *FCGR2/3* locus. Linkage Disequilibrium for SNPs and haplotypes in individuals without CNV. r^2 is shown for all combinations, which variant is linked to which variant is shown underneath. Values shown in bold are significantly different from 0 ($p < 0.05$). *FCGR2C*-ORF = classic *FCGR2C*-ORF haplotype vs. all other *FCGR2C* haplotypes. *FCGR2C*-NC-ORF = nonclassic *FCGR2C*-ORF haplotype vs. all other *FCGR2C* haplotypes. NA, not available, because the classic *FCGR2C*-ORF haplotype and nonclassic *FCGR2C*-ORF haplotype are mutually exclusive. Polymorphic amino acids are indicated by one-letter code.

TABLE 2 | Genotype and allele frequencies of functional genetic variants at the *FCGR2/3* locus, comparing KD patients of European descent with healthy controls of European descent.

Variant	Cases ($n = 405$)	Controls ($n = 919$)	Fisher	Single logistic regression (additive model)		Multiple logistic regression	
						All variants	2 variants
			P-value	OR (95%LL-95%UL)	*P*-value	*P*-value	*P*-value
CNR1 (*FCGR2C* + *FCGR3B*)							
0 copies	1	1		<2 vs. rest:			
1 copy	27	60		1.04 (0.66–1.66)	0.853	0.719	
2 copies	348	768					
3 copies	27	83		>2 vs. rest:			
4 copies	2	7	0.533	0.71 (0.46–1.10)	0.124	0.291	
CNR2 (*FCGR2C* + *FCGR3A*)							
1 copy	3	11		<2 vs. rest:			
2 copies	376	866		0.62 (0.17–2.22)	0.459	0.491	
3 copies	25	41		>2 vs. rest:			
4 copies	1	1	0.390	1.43 (0.87–2.37)	0.162	0.256	
CNR3 (*FCGR2C* + *FCGR3A*)							
2 copies	405	917		>2 vs. rest:			
3 copies	0	2	1.000	0.00 (0.00-inf)	0.973	0.973	
***FCGR2A* Q27W**							
QQ	289	713					
QW	108	194					
WW	8	12	**0.047**				
Allele frequency (W)	15.3%	11.9%		**1.35 (1.06–1.72)**	**0.014**	0.783	
***FCGR2A* H131R**							
HH	122	269					
HR	211	463					
RR	72	187	0.559				
Allele frequency (H)	56.2%	54.5%		1.07 (0.91–1.27)	0.408	0.857	0.927
***FCGR3A* V158F**							
0 V (F, FF, FFF, FFFF)	150	386					
1 V (V, VF, VFF)	205	403					
2 V (VV, VVF, VVFF)	47	128					
3 V (VVV)	3	2	**0.046**				
Allele frequency (V)	37.0%	35.5%		1.08 (0.91–1.28)	0.373	0.606	
***FCGR2C* promoter**							
0 2B.2	286	717					
1 2B.2	110	185					
2 2B.2	9	16					
3 2B.2	0	1	**0.017**				
Allele frequency (2B.2)	15.3%	11.5%		**1.37 (1.08–1.72)**	**0.009**	NE	
***FCGR2C* ORF/Stop/NC-ORF**							
0 ORF	283	721					
1 ORF	113	184					
2 ORF	9	13					
3 ORF	0	1	**0.005**				
0 NC-ORF	389	853					

(Continued)

TABLE 2 | Continued

Variant	Cases ($n = 405$)	Controls ($n = 919$)	Fisher	Single logistic regression (additive model)		Multiple logistic regression	
						All variants	2 variants
			P-value	OR (95%LL-95%UL)	*P*-value	*P*-value	*P*-value
1 NC-ORF	6	33					
2 NC-ORF	10	33	0.059				
Allele frequency (ORF)	15.7%	11.2%		**1.46 (1.16–1.85)**	**0.002**	0.093	**0.002**
Allele frequency (NC-ORF)	3.1%	5.2%		0.72 (0.51–1.02)	0.063	0.112	
Allele frequency (Stop)	81.2%	83.7%		0.88 (0.74–1.04)	0.136		
***FCGR3B* NA1/NA2/SH**							
0 NA1	158	373					
1 NA1	201	430					
2 NA1	45	114					
3 NA1	1	2	0.754				
0 SH	389	874					
1 SH	16	45	0.481				
Allele frequency (NA1)	36.2%	35.3%		1.01 (0.85–1.20)	0.933	0.537	
Allele frequency (NA2)	63.8%	64.7%		0.94 (0.80–1.12)			
Allele frequency (SH)	4.0%	4.9%		0.80 (0.45–1.43)	0.450	0.247	
***FCGR2B* promoter**							
0 2B.4	307	748					
1 2B.4	93	157					
2 2B.4	5	14	**0.043**				
Allele frequency (2B.4)	12.7%	10.0%		**1.29 (1.00–1.67)**	**0.047**	0.834	
***FCGR2B* I232T**							
II	322	697					
IT	76	201					
TT	7	21	0.359				
Allele frequency (T)	11.1%	13.2%		0.83 (0.64–1.06)	0.141	0.189	

For SNPs that are subject to CNV, several genotypes are pooled as indicated to combine all the different genotypes with the same copy number of 1 of the variants. For the tri-allelic haplotypes in FCGR2C and FCGR3B, this is done for two of the haplotypes separately. Fisher exact test was calculated on genotype frequencies as shown in the table. A single logistic regression analysis was performed for each (presumed) risk allele in an additive model. A multiple logistic regression analysis was performed on all variants (except the FCGR2C promoter haplotypes, which were left out of the multiple logistic regression analysis because of the near perfect LD with the classic FCGR2C-ORF haplotype) and on FCGR2A-H131R and classic FCGR2C-ORF alone. P-values < 0.05 are shown in bold.

Combined Analysis Reveals Both *FCGR2A*-131H and *FCGR2C*-ORF to be Significantly Associated With Susceptibility to KD

We performed a meta-analysis of the associations from both the case-control and familial TDT analyses, and we found the classic *FCGR2C*-ORF haplotype (rs759550223 and rs76277413, meta-P = 0.002) and the *FCGR2A* 131H (rs1801274, meta-P = 0.01) were both significantly associated with KD susceptibility (**Figure 4**).

mRNA for the FCGR2 Isoforms Is Upregulated in Acute KD Patients, in Contrast to the FCGR3 Isoforms

To determine whether alteration of expression levels of the low-affinity FcγRs plays a role in the pathophysiology of KD, we compared mRNA expression levels in KD patients in the acute and convalescent phase of the disease, using samples from a previous study (49). First, we compared Z scores for FCGR transcripts that were already present in the microarray for this study. In this analysis, we found FCGR2A, FCGR2B, FCGR3A, FCGR3B, and also FCGR1A, encoding the high-affinity FcγRI, to be all transcriptionally upregulated in acute KD (**Figure 5A**).

To confirm these findings and extend the analysis to FCGR2C, we then performed highly specific qPCRs for FCGRs on a selection of these patients from which RNA was still available. This confirmed that FCGR2A, FCGR2B and FCGR2C transcripts were all upregulated during acute KD (**Figure 5B**). FCGR3A was not differentially expressed between the acute and convalescent phase (**Figure 5B**) but FCGR3B seemed to be upregulated in the acute phase (**Figure 5B**). However, because acute KD could have resulted in a shift in leukocyte differentials and in our cohort a marked increase of neutrophil percentages was observed (data not shown), we applied a correction for percentages of different leukocyte subsets in the 100 patients for whom

leukocyte differentials were available. In the case of FCGR3B, a correction for neutrophil percentages (**Figure 5C**) showed that the apparent upregulation was the result of the relative increase in neutrophils during acute KD and does not reflect a true increase in transcription. On the other hand, expression levels of FCGR2A and FCGR2C were increased in acute KD even after correction for shifts in white blood cell distribution (**Figure 5C**).

Comparison for several genetic differences known to influence expression levels showed marked differences (**Figure 5D**), confirming earlier reports and the validity of our analysis.

DISCUSSION

In a comprehensive study using MLPA, we have analyzed the full collection of functionally defined SNPs and CNRs at the *FCGR2/3* locus at an unprecedented level of detail. We report extensive LD in this notoriously difficult gene cluster, as well as large ethnic variation in different European, African and Asian subpopulations. Our findings are in line with previously published allele frequencies and CNV in different populations for this locus (21, 44, 50) and extend these findings with additional variants and populations. Applying this as the reference dataset, previously reported genetic association studies may need to be re-evaluated.

This is the first study to illustrate the relevance of a more detailed reference for a pediatric vasculitis. KD has a ten-fold increased prevalence in Japanese and other Asian populations compared to children of European descent. In multi-ethnic GWAS studies, the association of *FCGR2A*-131H(rs1801274) with KD susceptibility was detected across KD cohorts of different ethnic backgrounds, indicating that this common variant is an independent susceptibility marker in all groups, including the Asian and European populations (6, 7). We now show that within the European cohorts, the classic *FCGR2C*-ORF haplotype (rs759550223 and rs76277413) may be the most strongly associated *FCGR* gene variant with KD susceptibility. Evidence from low LD ($r^2 = 0.08$) and conditional analyses identify the association of this classic *FCGR2C*-ORF haplotype to be independent of the previously identified *FCGR2A*-131H GWAS association. Interestingly, the classic *FCGR2C*-ORF, which is strongly associated with KD susceptibility in Europeans, was virtually non-existent in the Asian populations. This suggests that the increased prevalence of KD in Asian populations compared to European populations derives from factors other than the currently known genetic variation in *FCGR* genes.

The very strong LD of the classic *FCGR2C*-ORF haplotype with several other variants in the *FCGR2/3* locus means that the interpretation of associations with this locus are more complex than previously appreciated. Classic *FCGR2C*-ORF is in strong LD with three other variants: the 2B.2 promoter in *FCGR2C* (rs149754834), *FCGR2A*-27W (rs201218628) and the 2B.4 haplotype in *FCGR2B* (rs143796418). Hence, all these variants could tag the classic *FCGR2C*-ORF and were also significantly associated with KD susceptibility in a single logistic regression analysis. However, when we analyzed all variants in a multiple logistic regression analysis, we found the classic *FCGR2C*-ORF to be the strongest predictor of KD susceptibility.

TABLE 3 | Transmission disequilibrium test for the different variants at the *FCGR2/3* locus in a family-based association study.

Allele/haplotype (on 1 chromosome)	Allele frequency	# families*	*Z*	*P*-value
CNR1				
0 (deletion)	0.049	105	0.285	0.776
1	0.875	214	−0.065	0.948
2 (duplication)	0.074	133	−0.338	0.735
CNR2				
0 (deletion)	0.006	13	−0.277	0.782
1	0.976	61	0.378	0.705
2 (duplication)	0.018	48	−0.429	0.668
***FCGR2A* Q27W**				
Q	0.891	210	−0.328	0.743
W	0.109	210	0.328	0.743
***FCGR2A* H131R**				
H	0.575	431	2.750	**0.006**
R	0.425	431	−2.750	**0.006**
***FCGR3A* V158F**				
–	0.005	11	−0.302	0.763
F	0.642	395	0.483	0.629
FF	0.010	27	0.577	0.564
VF	0.006	18	−2.828	**0.005**
V	0.331	397	−0.088	0.930
VV	0.004	10	0	1.000
Promoter *FCGR2C*				
-	0.055	114	0.451	0.652
2B.1	0.748	349	−0.242	0.809
2B.1-2B.1	0.089	164	−0.818	0.414
2B.2	0.099	182	0.491	0.623
FCGR2C* ORF/Stop/NC-ORF*				
–	0.055	115	0.268	0.788
ORF	0.100	184	1.120	0.263
Stop	0.743	354	−0.241	0.810
NC-ORF	0.009	23	−1.460	0.144
Stop-stop	0.075	143	−0.477	0.633
NC-ORF-NC-ORF	0.007	19	−0.229	0.819
***FCGR3B* NA1/NA2/SH**				
–	0.053	108	0.186	0.853
NA1	0.362	389	0.490	0.624
NA1-NA2	0.051	96	−0.198	0.843
NA1-SH	0.009	21	0.218	0.827
NA2	0.508	396	−0.439	0.660
NA2-NA2	0.004	11	−0.905	0.366
SH	0.004	12	−1.155	0.248
Promoter *FCGR2B*				
2B.1	0.905	185	−0.563	0.574
2B.4	0.090	172	0.946	0.344
***FCGR2B* I232T**				
I	0.867	226	0.741	0.459
T	0.133	226	−0.741	0.459

**Number of informative families (i.e., at least one of the parents is heterozygous for the indicated allele or haplotype). Only alleles for which the number of informative families is >10 are shown. **ORF means classic FCGR2C-ORF haplotype, NC-ORF means nonclassic FCGR2C-ORF haplotype. Z; Z statistic, a positive Z indicates more transmission than expected, a negative Z indicates less transmission than expected, P indicates whether Z is significantly different from 0, P < 0.05 is considered significant.*

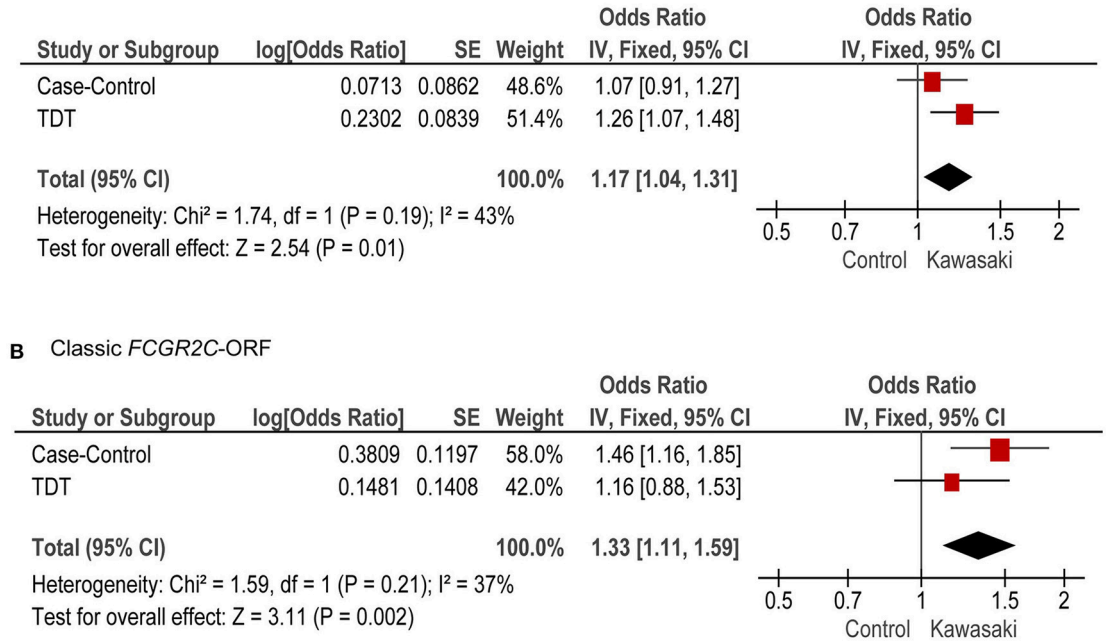


FIGURE 4 | Meta-analysis of case-control and TDT for *FCGR2A*-131H and the classic *FCGR2C*-ORF haplotype. Combined OR (95% CI) and *P*-values from the case-control study and TDT analysis, for *FCGR2A*-131H **(A)** and classic *FCGR2C*-ORF **(B)**.

The 2B.2 variant in *FCGR2C* was omitted from the multiple logistic regression analysis because of its near complete LD with classic *FCGR2C*-ORF. In fact, this variant can actually be only of biological relevance in the case of a classic *FCGR2C*-ORF haplotype, because with the other *FCGR2C* haplotypes, this 2B.2 promoter haplotype would reside in the promoter of an untranslated variant or *FCGR2C* (*FCGR2C*-Stop or nonclassic *FCGR2C*-ORF). It is unlikely that the tagging *FCGR2A*-Q27W SNP independently contributes to KD susceptibility, as it is a genetic variation for which a biological role has not been described (46). It lies outside the IgG-binding region of FcγRIIa and an analysis of expression levels revealed no influence on expression levels (**Figure S4**). However, genotyping the *FCGR2A*-Q27W SNP may be informative in genetic association studies, as it may be used as a tagging SNP for the classic *FCGR2C*-ORF as part of a susceptibility haplotype. The *FCGR2A*-Q27W SNP lies outside the copy number variable part of the *FCGR2/3* locus and is straightforward to genotype.

We did not find a significant association of CNV of the locus for any of the different CNRs that have been described. This is in contrast with an earlier report that described an association of CNV in *FCGR3B* and in *FCGR2C* with susceptibility to KD (51). In our opinion, analysis of CNV of *FCGR2C* without information on the *FCGR2C*-ORF variant is futile, as CNV of *FCGR2C per se* does not correlate with expression levels, normally being a pseudogene (i.e., *FCGR2C*-Stop). On the other hand, CNV in the *FCGR3B* does have a potential biological role, as we confirmed with our qPCR analysis, which showed a direct effect of CNV of the *FCGR3B* gene on transcript levels of FCGR3B. Nevertheless, CNV of *FCGR3B* was not associated with KD susceptibility in our cohorts.

Transcript levels of FCGR2A have previously been shown to be increased in KD patients compared to febrile controls (52), and we now show that mRNA levels of all FCGR2 isoforms, as well as FCGR1A1 [encoding FcγRI (CD64)], are upregulated during the acute phase of KD, compared to paired convalescent samples of the same patients, which further underscores the importance of FcγRs in KD.

A striking finding of our study is the lack of a significant association of *FCGR2A*-131H in the case-control study, contrasting our previous GWAS findings (6). This discrepancy was not explained by a difference in allele frequency in the case group, but by a difference in allele frequency between the control groups tested. Both control groups were randomly selected individuals of European descent. A remarkable difference between the two control groups was that the control group of the GWAS consisted mainly of individuals from the United Kingdom, which in the present study have a significantly lower prevalence of the *FCGR2A*-131H than the other European groups (**Table S3**). Apparently, even within the European population, the selection of the control group may influence the results of association analyses. Although both control groups were randomly selected, we believe that the group used in the current study is more representative of the background population, since it consists of more controls from the countries of origin of the patients. Nevertheless, even with the new control group, in a combined meta-analysis with our TDT analysis, *FCGR2A*-131H was still significantly associated with KD susceptibility.

In addition to small differences within the European population, of more relevance were the significant differences in allele frequencies at the *FCGR2/3* locus between the different ethnic groups. Our MLPA assay enabled us to look at the

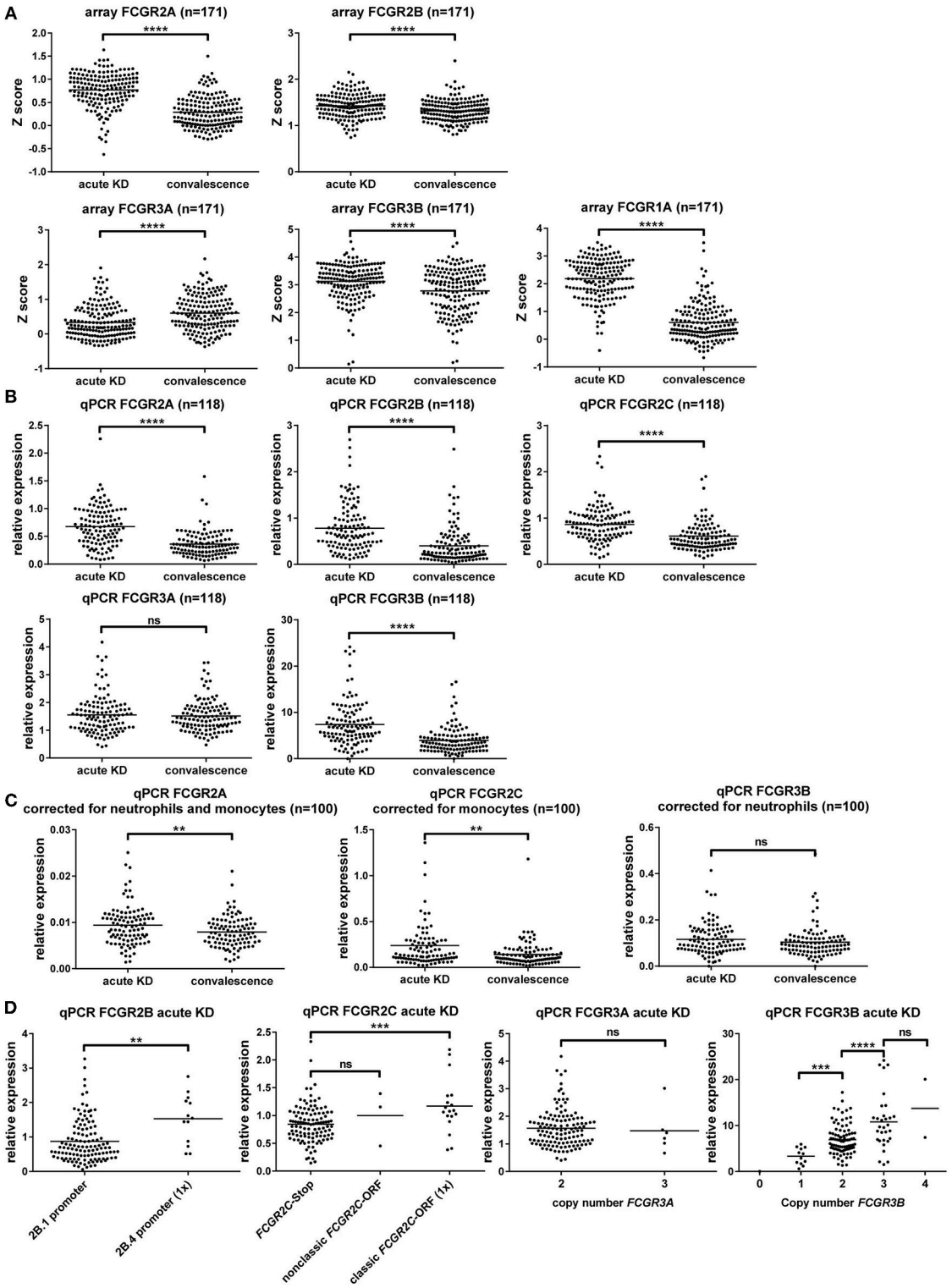


FIGURE 5 | Gene expression analysis of FcγRs shows upregulation of FcγRI and FcγRII, but not FcγRIII during acute KD. **(A)** Difference in expression intensity of various FCGR transcripts, as determined by RNA microarray, shown as Z scores (higher score indicating higher expression), in 171 subjects with KD in the acute and convalescent phase of the disease. **(B–D)** Relative expression of different FCGR transcripts detected by qPCR on whole blood, corrected for housekeeping genes GUS and GAPDH, as compared to one randomly chosen sample in the convalescence phase of KD. **(B)** Dot plots showing a comparison of the acute and convalescent phase of KD in 118 patients. **(C)** Dot plots showing a comparison of the acute and convalescent phase of KD for transcripts of FCGR2A, FCGR2C,

(Continued)

FIGURE 5 | and FCGR3B in 100 patients for which WBC differentials were known, after correction for the main cell type that expresses the transcript. **(D)** Comparison of genotypes for the expression of various transcripts in 135 patients with acute KD. FCGR2B: patients with only the 2B.1 promoter ($n = 122$) or with 1 copy of the 2B.4 promoter ($n = 13$) in *FCGR2B*. FCGR2C: patients with the *FCGR2C*-Stop haplotype ($n = 114$), patients with 1 copy of the classic *FCGR2C*-ORF haplotype ($n = 18$), patients with 1 or 2 copies of the nonclassic *FCGR2C*-ORF haplotype ($n = 3$). FCGR3A patients with 2 copies ($n = 129$), or 3 copies ($n = 6$) of the *FCGR3A* gene. FCGR3B: patients with 0 copies ($n = 1$), 1 copy ($n = 12$), 2 copies ($n = 89$), 3 copies ($n = 31$) or 4 copies ($n = 2$) of the *FCGR3B* gene. ns: non-significant; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ as determined by paired *t*-test or Wilcoxon matched-pairs signed rank test **(A–C)** or students *t*-test or Mann Whitney test **(D)**.

distribution of *FCGR2C* haplotypes in African, European and Chinese populations. We show that MLPA reliably distinguished the classic *FCGR2C*-ORF from the nonclassic *FCGR2C*-ORF haplotype that does not result in expression of FcγRIIc. Theoretically, only minimal errors in haplotype calling can occur for *FCGR2C* with the MLPA methods (calculated error rate of only 0.1%, **Table S4**), whereas Illumina whole-exome sequencing was unable to detect the rs759550223 SNP of the classic *FCGR2C*-ORF haplotype in all three individuals with this haplotype among ten individuals tested in total (error rate 30%) (18).

The classic *FCGR2C*-ORF haplotype is virtually absent from the Asian population, whereas in the African population, the non-expressed nonclassic ORF was much more prevalent than the classic *FCGR2C*-ORF. The absence of the classic *FCGR2C*-ORF in the Asian population is of particular interest because of the fact that there is a striking difference in the incidence of KD between children of Asian (69–308 per 100,000 children <5 years of age) (53) and of European descent (4–15 per 100,000 children <5 years of age) (54–56). Clearly, the *FCGR2C*-ORF is only a risk factor for KD susceptibility in European subjects, and cannot account for the increased incidence of KD in Asian children.

A potential limitation of our MLPA technology lies in the uncertainty of allocating the promoter haplotypes 2B.2 and 2B.4 to either *FCGR2B* or *FCGR2C*, but data previously generated by us and others (5, 36, 57, 58) show that our allocation approach is accurate in >95% of European individuals with at least one of the rare variants 2B.2 or 2B.4. The majority of individuals does not carry a rare variant and these individuals will be 100% accurately genotyped by MLPA.

Detailed knowledge of genetic linkage in IgG receptors has major implications for every other study on associations of *FCGR2/3* polymorphisms with disease or therapeutic efficacy. For example, many studies investigating associations with therapeutic efficacy of therapeutic antibodies against cancer have found an association with the *FCGR3A*-158V variant (rs396991) (13–15, 59), which we now show to be in moderate LD with the classic *FCGR2C*-ORF ($r^2 = 0.24$). Since the classic *FCGR2C*-ORF haplotype leads to expression of the activating FcγRIIc on NK cells, neutrophils, monocytes (**Figure 2**) and macrophages (17), it may contribute to killing of tumor cells by antibody-dependent cellular cytotoxicity by these cells, and could potentially be a stronger predictor of treatment success.

In conclusion, we have reported a novel association of the classic *FCGR2C*-ORF variant (rs759550223 and rs76277413) with susceptibility to KD in European patients, independent of the *FCGR2A*-131H (rs1801274), which is a separate susceptibility marker. Upregulation of the transcripts for both activating receptors encoded by these genes (respectively FcγRIIc and FcγRIIa) during acute KD further indicates their importance in KD pathophysiology. FcγRIIa and FcγRIIc are co-expressed by two circulating cell types, monocytes and neutrophils. Both cell types are actively recruited to arterial lesions in KD patients. Our data support a central role of the activating IgG receptors on these cell types in the pathophysiology of KD, whereas the SNPs in the inhibitory FcγRIIb were not associated. This suggests that inhibiting the function of activating FcγRs (which is a possible working mechanism of IVIg, the first-line treatment in KD) may be an important treatment goal in patients with this pediatric vasculitis during the acute phase of the disease.

AUTHOR CONTRIBUTIONS

SN and CT performed experiments, analyzed data, wrote the manuscript and designed research. WB discussed data and designed research. MT performed statistical analysis. JG, LH, EP, AN, and JvdH performed experiments and analyzed data. RY, ML, VW, DB, A-LP, JE, RC, CS, JB, KF, and CvdS provided samples. TvdB, SD, and MH supervised research. MdB discussed data and designed research. TK supervised the study, wrote the manuscript and designed research. All authors contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00185/full#supplementary-material

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REVIEW
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Inside-Out Control of Fc-Receptors

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Receptors recognizing the Fc-part of immunoglobulins (FcR) are important in the engagement of phagocytes with opsonized micro-organisms, but they also play a major role in the pathogenesis of chronic inflammatory diseases. Different FcRs are specifically recognizing and binding the different classes of immunoglobulins, transmitting different signals into the cell. The function of IgG (FcγR's) and IgA (FcαR) recognizing receptors is controlled by cellular signals evoked by activation of heterologous receptors in a process generally referred to as inside-out control. This concept is clearly described for the regulation of integrin receptors. Inside-out control can be achieved at different levels by modulation of: (i) receptor affinity, (ii) receptor avidity/valency, (iii) interaction with signaling chains, (iv) interaction with other receptors and (v) localization in functionally different membrane domains. The inside-out control of FcRs is an interesting target for novel therapy by therapeutical antibodies as it can potentiate or decrease the functionality of the response to the antibodies depending on the mechanisms of the diseases they are applied for.

Keywords: inside-out control, immunoglobulins, priming, activation, phagocytes, Fc-receptors

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INTRODUCTION

Immunoglobulins have evolved during evolution as a link between the antigen-specific adaptive immunity and the molecular pattern driven innate immune system. These molecules contain an antigen-specific variable region brought about by gene rearrangement in B-cells. The underlying mechanisms for this rearrangement has been reviewed elsewhere (1). For the purpose of this review it suffices to say that this variable part enables specific binding to antigens beyond the common patterns recognized by the innate immune system. After the antibody binds the antigen via the variable regions, the constant region of the immunoglobulins denoted as Fc part can be recognized by other immune cells and facilitate the immune response (2–4).

The Fc-part of immunoglobulins is relevant at three different levels. First of all the Fc-part determines the human (sub)class of the immunoglobulin: IgA (IgA1 and IgA2), IgD, IgE, IgG (IgG1, IgG2a, IgG2b, IgG3, and IgG4) and IgM. During gene rearrangement the B-cell determines, guided by cytokines in its environment, which (sub)class of immunoglobulin is later produced by the respective plasma cells. The second level is the propensity of some classes of immunoglobulins to activate and fixate complement, which greatly enhances the recognition of antigens through recognition of C3b and C3bi by complement receptors (CR1/CD35 and CR3/CD11b) on phagocytic cells. The binding of immunoglobulins and complement fragments to antigens is generally referred to as opsonization.

Finally, the third level by which the Fc-part of immunoglobulins is important, is the recognition by specific Fc-receptors. These Fc receptors are mainly expressed by effector cells of the innate immune response [for excellent reviews see (2–7)].

Every class of immunoglobulins has specific receptors that can recognize these subclass specific Fc portions. These receptors are indicated by Greek letters: FcγR for IgG, FcαR for IgA, FcεR for IgE, FcδR of IgD and FcμR for IgM. Apart from these receptors also the neonatal FcR (FcRn) is expressed by stromal cells and is involved in transfer of immunoglobulins from blood to the tissue (8). Some of the immunoglobulins have more receptors with various affinities for the different subclasses as IgG comes in 5 subclasses (IgG1, IgG2a, IgG2b, IgG3, and IgG4) and IgA in two (IgA1 and IgA2). The situation with IgA is even more complex as the molecules are found as both monomers and dimers, and on mucosal surfaces as dimers with a J-chain and secretory component. The latter form of IgA is referred to as secretory IgA, which can still be recognized by FcαR (2, 3). However, additional receptors for the secretory component can modify the binding characteristics of secretory IgA (9).

The FcRs are under tight control as the immune system should evoke a balanced response to invading micro-organisms as well as to signals that can lead to aberrant activation of the immune system such as seen in chronic inflammatory disease including autoimmune disorders (10). Too much activation leads to collateral damage to the host tissue, whereas too little activation can lead to infections. The control of the function of the FcRs is the subject of this review.

FC-RECEPTOR FUNCTIONING IN THE INNATE IMMUNE RESPONSE

The best known function of FcRs is their role in phagocytosis and killing of opsonized targets. Phagocytosis refers to the process of specialized cells of the immune system that can engulf and take up targets into intracellular organelles called phagosomes (11). These phagosomes are closed and do not have any link with the extracellular milieu. In these organelles the cells can induce a very hostile environment by which the phagocytosed target is killed. This is mediated by multiple processes: fusion of granules filled with cytotoxic proteins, enzymes and peptides, production of toxic oxygen intermediates by a membrane bound NADPH-oxidase, and a lowering of the pH in the phagosome (12).

The fusion of the granules with the phagosome is often referred to as degranulation. This fusion of the phagosome with the granules leads to the formation of so-called phagolysosomes in which the actual killing of microbes takes place. Degranulation is not only into these phagolysomes, but occurs also by fusion of the granules with the plasma membrane. Then the cytotoxic components are liberated into the extracellular space, where they are involved in killing of the targets outside the cell. It will be clear that this extracellular process comes with a cost: damage to the healthy host tissues (13). This process of extracellular killing is also employed by eosinophils and macrophages killing large multicellular targets such as helminths; targets several times larger than the immune cells. Patnode et al. (14) describe clear swarming behavior of eosinophils interacting with helminths that leads to a "together we are strong" type of killing. There is a clear synergism in killing mediated by degranulation and the activation of the NADPH-oxidase; the other major mechanism involved in killing of micro-organisms by phagocytes (15).

It will be clear from the above that FcRs are very important in the interaction of the host with pathogens. This review will focus on two classes of FcRs as these are important in phagocytosis and killing of micro-organisms: FcγR and FcαR. Six genes encode FcγR's in humans: FcγRI (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIC (CD32C), FcγRIIIA (CD16A), and FcγRIIIB (CD16B) (4). These receptors are expressed by various immune cells in different combinations and have different affinities for the different IgG subclasses (4). There are several IgA receptors: FcαRI (CD89), transferrin-receptor-1 (CD71), asialoglycoprotein-receptor (ASGPR/), Fcα/μR, FcRL4, and DC-SIGN/SIGNR1 (2). However, the best studied in the context of immune function and phagocytosis is FcαRI (CD89) and, therefore, we will focus on this IgA-receptor in this review.

Signal Transduction

Signal transduction of FcRs has been studied in detail and reviewed by Bournazos et al. (16). In short, broadly three modules of signaling are found for these receptors: (1) Direct signaling by the receptor itself (CD32s), (2) Via an accessory common FcRγ-chain (CD64 and CD16A and CD89), and (3) indeterminate signaling because of the absence of an intracellular tail [Glycosylphosphatidylinisotol (GPI) anchored CD16B].

Direct Signaling

Direct signaling by CD32 is mediated by immunoreceptor tyrosine-based activation motif (ITAM/CD32A and CD32C) (17) and by immunoreceptor tyrosine-based inhibitory motif (ITIM/CD32B) (18). These motifs determine whether the receptors are activating or inhibitory. It is important to emphasize that signaling starts by cross-linking of the receptor leading to activation of phosphatases such as SHP and SHIP, and members of the src-family of tyrosine kinases (19–21). This leads to phosphorylation of the important tyrosine residues in the ITAM/ITIM motifs from where various signaling cascades are initiated. Phosphorylation of ITAMs lead to activation of the cells (22), whereas phosphorylation of ITIMs lead to cell inhibition (23). The mechanisms involved in the control of CD32B have been excellently reviewed by Getahun and Cambier (24).

Signaling via an Accessory Common FcRγ-Chain

Signaling via an accessory common FcRγ-chain is also mediated by ITAM motifs present in the γ-chain. Here the main signaling is not mediated by the intracellular tail of the FcR itself, but by the FcRγ-chain that is associated with the receptor. This mode of action is found for CD16A, CD64, and CD89. Similar signals are initiated compared to direct signaling from the receptor (25–27).

Indeterminate Signaling

Indeterminate signaling seems to be the characteristic of CD16B expressed at high levels on human neutrophils. This receptor lacks both an intra-cellular portion and a transmembrane domain as it linked with the membrane with a GPI-linkage (28). However, it is likely too simple to consider this receptor as

signaling dead. Various studies indicate that cross-linking CD16B evokes signaling characterized by e.g., changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (29). The general idea is that cross-linking leads to an engagement with other receptors that in turn activate a signaling cascade. The identity of such a receptor in CD16B signaling remains to be defined, but studies indicate that integrins and integrin associated proteins might be candidates(30). Such mechanism *in trans* can also be part of signaling through the other signaling FcRs (30, 31). This paradigm will be discussed in more detail later in the review.

Most of the IgG and IgA receptors exhibit a low or intermediate affinity for their monovalent ligands with an exception for FcγRI/CD64 that has a high affinity for monomeric IgG. The low affinity receptors do not bind to monomeric ligand or this binding is so low affinity that it is difficult to determine *in vivo* (32). The consequence of this low affinity is that these receptors only bind to multivalent ligand such as found in immune complexes as well as Ig coated surfaces such as found on opsonized micro-organisms(3). This in contrast to FcγRI that is always bound to IgG, but that interestingly does not lead to appreciable signaling (33).

An additional mode of control of FcRs is the multimerisation of the receptor into clusters at the cell membrane by which their valency increases (34). Modulation of this valency is a means by which the cell can facilitate the interaction with Ig-coated surface.

THE CONCEPT OF INSIDE-OUT CONTROL

The Concept of Inside-Out Control Identified in Integrin Function

The concept of inside-out control of immune receptors was first put forward for the function of integrins (35). It basically refers to an increase in receptor affinity, valency and/or function induced by intracellular signals initiated by heterologous stimuli. A very clear example is the finding that a mutation of the Kindlin-3 gene in patients with leukocyte adhesion deficiency III leads to a complete block in the functionality of β2 chain containing integrins LFA-1, Mac-1 and p150.95 (36). The genes and expression of these receptors are normal, but functionality is lacking leading to a clinical phenotype reminiscent of LAD1 where the β2-chain (CD18) gene is mutated and expression of the CD18 integrins is absent (37). A similar situation is found for the fibrinogen receptor (αIIb/β3) that is dysfunctional in these Kindlin-3 deficient patients. The molecular mechanisms underlying inside-out control of integrins is excellently reviewed by the group of Ginsberg et al. (35, 38).

Inside-Out Control of FcR

Next to integrins various studies show that also FcγR's and FcαR are subjected to inside-out control (39–43). In contrast to integrins where a consensus is present that this mechanism is important, this concept has not yet been generally accepted for FcR function. The main problem with the latter receptors is that many immune cells express multiple FcRs for the same ligand Ig which makes the study of individual receptors difficult. The studies that have focused on inside-out control of specific FcRs have either been performed with cells endogenously expressing only a single Fc-receptor or cell models dependent on cytokines exogenously expressing single Fc-receptors (39–42, 44).

FcγRII

An excellent cell to study the inside-out control of FcγRIIA is the human eosinophil. This cell isolated from the blood of healthy control only expresses this FcγR. Early work showed that eosinophils carefully isolated in a non-primed fashion hardly bind beads coated with human IgG while they clearly express FcγRII as visualized in FACS based assays (42). Short term pre-incubation with cytokines such as IL-5 and GM-CSF or chemotaxins such as platelet-activating factor (PAF) lead to clear binding of the cells to these Ig-coated particles, whereas the expression of the receptor on the cell surface was unaltered. This model also allowed the manipulation with different pharmacological inhibitors to find out which signaling models are important in this inside-out control (44). These experiments identified that the MEK-MAP-kinase based signaling in these cells is important as MEK inhibitors clearly block the interaction of pre-activated eosinophils with Ig-coated particles (44). These findings basically imply that different cytokines differentially engaging different signaling pathways can steer the inside-out control of FcγRII: those that engage MEK-MAPK such as IL-5 steer the function of FcγRII, whereas those that more engage PI-3K and p38 such as IL-4 more activate FcαR [see below and (44)]. Similar experiments are very difficult to perform with neutrophils because of the high co-expression of FcγRIII (CD16B). It should be emphasized that Huizinga et al. have shown that FcγRII is also the main signaling IgG-receptor in neutrophils (45) and most likely controlled by a similar signaling module as operational for FcαR (42). However, direct experimental proof is lacking. Interestingly, Aleman et al. (46) described the importance of FcγRIIIB in netosis of neutrophils supporting the concept of FcγRIIIB as a signaling receptor.

FcαRI

This receptor is expressed by multiple immune cells including eosinophils. It is, however, important to mention that FcαRI on eosinophils is heavily glycosylated and behaves differently in SDS-PAGE gels when compared with the receptor present in e.g., neutrophils (47). Comparable with serum-IgG coated beads, only (cytokine) primed eosinophils interact with IgA-coated beads (44). However, for FcαR mediated interaction between IgA-coated targets and primed eosinophils the PI-3-kinase signaling pathway is important. This has important consequences as cytokines such as IL-4 that primarily engage this pathway without apparent activation of the MAP-kinase pathway only induce binding of eosinophils with IgA coated targets and not IgG coated targets (44). Interaction with IgG coated beads is not sensitive for (cytokine) priming, likely because FcγRIIIB that is highly expressed by neutrophils can facilitate the interaction with IgG coated beads.

These findings have consequences *in vivo* as differential priming with different mediators at different times and places will determine whether innate immune cells will engage with opsonized particles. It is important to emphasize that eosinophils isolated from patients with allergic diseases exhibit a primed

phenotype with respect to binding to IgG and IgA coated beads (48). This implies that these cells have engaged with Th2 driven cytokines and other mediators leading to long term priming of the cells as the primed phenotype persisted during the whole isolation procedure *ex vivo*. Thus, the FcRs retain their primed phenotype for a long time *in vitro*. The situation *in vivo* is less clear as the group of Chilvers et al. put forward the hypothesis that part of the primed phenotype of granulocytes associated with primed FcRs deprimes in the lung *in vivo* (49, 50). This concept, however, has been tested for neutrophils but not for eosinophils. The expression of multiple FcRs on neutrophils precludes a simple testing of the hypothesis that depriming leads to deactivated FcRs on granulocytes.

The mechanisms underlying inside-out control are multiple, complex and cross-interacting. They can be at the level of the receptor itself, associated signaling partner molecules, clustering of homologous and heterologous receptors allowing activation in *trans* and last but not least changes in organization of plasma membrane specialized areas such as lipid rafts and caps.

The functionality of FcRs expressed on the plasma membrane can be accomplished at different levels: (1) changes in valency (multiple receptors are engaged by multivalent ligands on opsonized surfaces (see **Figure 1**), and (2) changes in affinity of single receptors for their ligands.

The valency of receptors is very important as the consensus in the field is that cross-linking of receptors by multiple ligands on the opsonized surface is the main trigger for activation through FcRs (34). It is generally believed that tyrosine kinases binding the one FcR cross-phosphorylate tyrosine residues in ITAM's/ITIM's of the adjacent FcRs. This then initiates the signaling cascades leading to the activation of the downstream functions. So these receptors have to come together in order to be able to signal. Cross-linking by itself seems to be sufficient for signaling as artificial cross-linking by receptor antibodies leads to phosphorylation of the receptors and induction of signaling (29, 51). However, artificial cross-linking does not completely recapitulate the activation induced by natural ligand. This is nicely illustrated by the finding that cross-linking of FcγRIIIB (CD16B) that does not have any intracellular tail leads to changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) whereas no signaling motif is present in this receptor. Although it might be that cross-linking of FcγRIIIB engages FcγRIIA through the Fc-portion of the CD16 antibody. In addition, such changes in $[Ca^{2+}]_i$ are not necessarily induced by natural ligand in the form of immune complexes (45) or serum-opsonized particles (52). Thus, caution should be taken to apply artificial cross-linking of the receptor as surrogate for FcR signaling. It is also difficult to test the hypothesis that an increase in valency (receptor clustering) is sufficient for FcR signaling as it is difficult to accomplish this without additionally affecting the receptor affinity for its ligand.

The affinity of low-affinity receptors for their ligands is difficult to determine as monomeric ligand does not bind with sufficient affinity even after inside-out activation. This makes sense as the immune system ideally does not want to interact with monomeric Ig's in blood and mucosal tissues. Therefore, it is very difficult to study valency and affinity of FcRs as two independent mechanisms and, therefore, the functionality of FcR in the remainder of this review is the resultant of both increased valency and affinity.

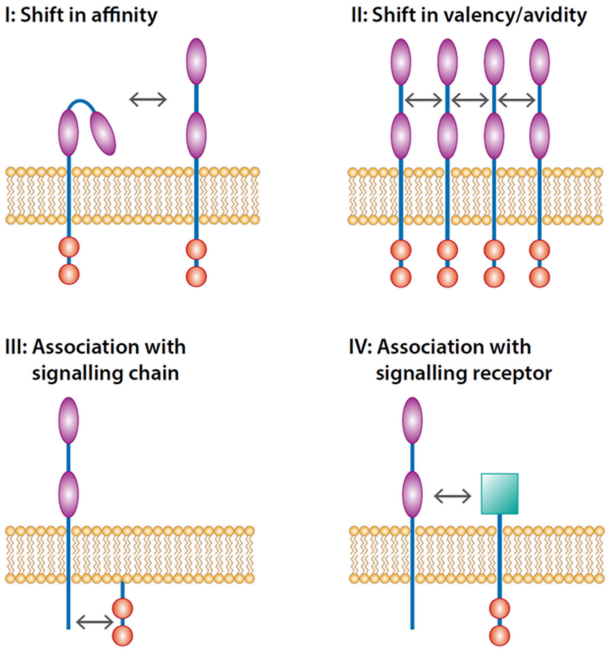

FIGURE 1 | Levels of inside-out control of FcRs . Inside-out control can be accomplished at four levels. The first level is modulation of affinity of single receptors opening up the binding domain(s) of the immunoglobulin domains (illustrated by ⬬) facilitating the activation of the receptor which in turn signals through signaling motifs (illustrated by ●) present in the receptor. The second level is induction of lateral movement of the receptor increasing the valency of the receptor. The third level is the interaction with signaling chains such as FcRγ-chain that transmit the signal-tranduction pathways. The fourth level is the trans-activation through an associated heterologous signaling receptor (illustrated by ■).

THE IMPORTANCE OF THE INTRACELLULAR TAIL OF FcRs IN INSIDE-OUT CONTROL

In order for inside-out control to be affecting the functionality of FcRs these signals should converge at the intracellular tail of the FcRs such as also found for integrins (38). This concept is studied in more molecular detail for two FcRs: FcαRI (CD89) and FcγRI (CD64) (39–41, 53, 54).

The Importance of Serine263 in the Intracellular Tail of FcαRI

Initial studies indicated that several kinases, PI-3-kinase, MAP kinase and p38 were critically involved in the activation of the functionality of FcαRI (40, 41, 48). This was found both in primary cells (leukocytes) as well as cell lines ectopically expressing this receptor. Inhibitors of these kinases modulated the activation FcαRI even in the absence of the accessory

common Fcγ-chain. This implies that phosphorylation is involved in the direct control of the functionality of FcαRI. However, there were no clear consensus motifs present in the intracellular tail that are preferentially phosphorylated by any of these kinases. In depth analysis of the receptor led to the conclusion that FcαRI expressed in resting cells was constitutively phosphorylated and thus that kinase activity was already found in the cytosol of resting cells (41). This led to the concept that FcαRI is actively suppressed in its function by constitutive phosphorylation of the receptor. By studying FcαRI receptor mutants transfected into cytokine-dependent Ba/F3 cells it was found that a serine residue at the 263 position in the intracellular tail is essential for the functionality of FcαRI in the context of binding to IgA-coated beads. Mutation of the serine residue to alanine led to a constitutively active receptor supporting the concept that an active kinase is important in keeping the receptor in a non-functional state. This hypothesis was supported by the finding that the S>D mutation, introducing a negative charge at the 263 position, lead to a non-functional receptor as if it was constitutively phosphorylated (41).

Ten Broeke et al. recently provided evidence that the identity of this constitutively active kinase is glycogen synthase kinase-3 (GSK-3) (43), a kinase that is constitutively active in resting cells such as leukocytes and its activity is inhibited by phosphorylation (43). Interestingly, such phosphorylation can be mediated by cytokine-induced activation of the PI-3K and protein kinase-Cζ (PKCζ) -axis (43). This leads to a model were the function of FcαRI is actively suppressed by phosphorylation by GSK-3 in unactivated cells. Cytokine-induced activation of PI-3K followed by activation of PKCζ leads to phosphorylation and inactivation of GSK-3. This in turn leads to dephosphorylation and activation of FcαRI. It is still unclear at which level the control of dephosphorylating of the receptor is achieved. It might be that a constitutive active phosphatase dephosphorylates the receptor or that such enzyme is actively controlled by inside out signals such as found for FcγRI (39). Unfortunately, a similar concept has not been developed in any detail for the inside-out control of FcγRII.

Mechanism of Inside-Out Control of FcγRI (CD64) Functionality: The Tail and Importance of Phosphatase Activity

The situation is different with FcγRI as this is a high affinity receptor able to bind to monomeric IgG. The general idea is that this receptor is always occupied by ligand under conditions such as found in the plasma. Nonetheless, several indications in studies by the group of Leusen et al. (55) provided evidence that this receptor irrespective of bound monomeric IgG can still bind to immune complexes. Only this latter binding is sensitive for inside-out control. The receptor ectopically expressed in hematopoietic cell lines is sensitive for inside-out signaling. The concept arising from this study is that the phosphatase PP2A is the driving enzyme involved in dephosphorylating the receptor and thereby activating its functionality (39). Here again the phosphorylated receptor has a low functionality and dephosphorylation leads to activation. The underlying mechanisms are not yet completely understood, but recently Brandsma et al. have described that inside-out control of FcγRI is at least in part mediated by lateral movement of the receptor in the membrane (54). It is tempting to speculate that this modulation of movement will be important for the control of valency of this receptor.

The Intracellular Tail and the Inside-Out Control of FcγRII (CD32)

The importance of the intracellular tail of FcγRII comes from experiments in cell lines showing that ectopically expressing tail-less version of FcγRIIA/B is accompanied by a blunted signaling response (17). To test the hypothesis that the tail of FcγRIIA is also important in phagocytes Clark et al. (56) transduced neutrophils with a cell permeant peptide encompassing the intracellular tail of FcγRIIA. They could show that this peptide decreased Ca^{2+} signaling as well as formation of phagolysosomes in human neutrophils.

It is clear that the intracellular tails of FcγRIIA/B, FcγRIIIA, and FcαRI are important for signaling. However, tail-less mutants co-expressed with other receptors such as integrins are still able to transmit signals indicating the intimate cross-talk between these receptors and alternative signaling chains (57).

INSIDE-OUT CONTROL AND RECEPTOR INTERACTIONS

The view that only valency and affinity are important for the inside-out function of Fc-receptors is too simple. The complexity of the ligands (uni/multi valent, fixed complement etc.), immune complexes and opsonized microbes, is very relevant. Here additional proteins and other ligands are present/expressed that can bind to a multitude of additional receptors on innate effector cells e.g., integrins, Toll-like receptors, glucan receptors, complement receptors etc. It is to be expected that differential inside-control mechanisms will control some if not all of these receptors. It will be clear that the net result of all these interactions will lead to a very complex situation that is difficult to understand from the view of individual receptor function.

FcγR/FcγR Cross-Talk

Most innate effector cells express multiple FcRs and most multiple FcγR's. Monocytes and macrophages express FcγRI, FcγRII, and FcγRIII, neutrophils FcγRIIA and FcγRIIIB. Eosinophils only express FcγRIIA and maybe FcγRIIB/C. Cross-talk between FcγRII and FcγRIIIA/B has been suggested by various experiments. Co-crosslinking of FcγRIIA and FcγRIIIB leads to a clear activation of neutrophils characterized by changes in $[Ca^{2+}]_I$ and downstream functions (29). On NK-cells FcγRIIA and FcγRIIIA cross-modulate their functions (58). It is tempting to speculate that subtle changes in inside-out control of these individual receptors will influence the end result of co-activation.

FcR-Integrin Cross-Talk

Several studies have shown that the interaction of primary cells expressing both integrin receptors and FcRs with opsonized targets is characterized by a clear cross-talk (30, 59–61). Again

this is best shown in cells that express relatively little different FcRs to exclude interference of above mentioned FcγR/FcγR interactions. Again eosinophils are an interesting cell model as they only express FcγRIIA as activating FcγR. It has clearly been shown that a synergism is present when a surface is expressing integrin ligands such C3bi (ligand of Mac-1/CR3) together with Ig's. Van der Bruggen et al. have shown that yeast opsonized with both ligands is superior when compared with yeast only coated with Ig's or complement (60). However, a trivial explanation might be that the affinity/avidity of the opsonin receptors might be higher when both ligands are present.

Ortiz-Stern et al. have described the importance of cross-linking of FcγRIIIB on neutrophils in modulation of β1-integrins whereas cross-linking of FcγRIIA and FcγRIIIb both lead to activation of β2-integrins (62). More of this type of cross-talk between FcRs and integrins has been reviewed by this group (59). Relevant for this concept is the finding that a genetic polymorphism in the FcγRIIIb gene affects the interaction of this receptor with FcγRIIA and Mac-1/CR3 (CD11b/CD18) (63).

FcR-TLR Cross-Talk

Apart from functional interactions between opsonin and integrin receptors, the function of FcRs is also modulated by multiple other receptors. An important class are the pattern recognition receptors such as toll-like receptors. These receptors can engage with FcR signaling by physical interaction as well as through signaling after ligand binding (64). Indeed, co-immunoprecipitation studies in murine neutrophils have shown that TLR4 (LPS-receptor) physically interacts with FcγRIII upon binding to its ligand LPS (64). It is good to emphasize that there are marked differences in FcRs and Toll-like receptors between mouse and man (4, 65).

For cross-talk between FcR and TLR both receptors do not need to physically interact as the main signaling pathways induced by TLR activation, NFκb, MAP-kinases and PI-3 kinase, are important in inside-out control of FcγRIIA and FcαRI. More of these interactions between FcR and TLR have been excellently reviewed recently (66).

Inside Control of FcRs by Other Receptors or Signaling Molecules

Apart from TLR's there is a whole range of cytokine/chemokine receptors and glucan receptors that all have in common that they engage in signaling pathways important for inside-out control of FcRs. It will be clear that these signals control the interaction between innate effector cells and their targets. This mechanism has been known for a long time and was generally referred to as priming: a process that does not induce a certain cell function by itself but greatly enhances this response to a (heterologous) agonist (67). Particularly, cytotoxic responses are sensitive for such priming responses that act as "safety locks" to prevent aspecific activation of inflammatory cells. Part of such a priming response is mediated by the interaction of FcRs with function modulating membrane proteins.

Not many membrane receptors/chains other than FcR-γchain, additional FcRs or integrins have been described to be involved in the functionality of FcRs. The correct expression of FcRs is dependent on the presence of β-2 (CD18) integrins. Kindzelskii et al. have described the aberrant capping responses of membrane proteins including FcγRIII and the urokinase receptor in patients with leukocyte adhesion deficiency I (LADI) (68). The data imply that a physical cross talk between integrins and FcRs is part of the correct functioning of FcRs (57). The reverse has not been published.

Next to these aforementioned binding partners periplakin has been implicated in the regulation of function of FcγRI (69). The authors described that periplakin was important in receptor recycling as well as ligand affinity. Periplakin has also been implicated in the control of G-protein coupled receptors, which might important for the signaling of FcR in trans (70) (see below).

INSIDE-OUT CONTROL AND GLYCOSYLATION OF FC-RECEPTORS AND IMMUNOGLOBULINS

In recent years another concept of inside-out control has emerged. It turned out that differences in glycosylation of Fc-receptors has a major impact on their functionality as has recently been reviewed by Hayes et al. (71). This mode of control is nicely illustrated by Patel et al. (72) showing that the function of FcγRIIIA on NK-cells is dependent on its glycan composition. This implies that post-translational processing of FcR is of importance for their functionality on the cell membrane (73). It is not only the glycosylation of FcRs that is important, but also the glycosylation of the different Ig's as large differences are found between the functionality of certain Ig's depending on their N-glycan content (74, 75). Interestingly, also anti-inflammatory characteristics of IgG can be attributed to differences in glycosylation (76). In conclusion, by affecting glycosylation of both FcRs as well as Ig's immune cells can steer the immune response. This has major consequences for designing therapeutical antibodies (77).

INSIDE-OUT CONTROL AND SIGNALING IN *TRANS*

As mentioned before FcRs can signal through their intracellular tail and/or through an accessory FcRγ chain constitutively associated with the receptor. A third mechanism is activation in trans through heterologous receptors associated with the FcRs only after (pre)activation. This concept of signaling in *trans* has been identified many years ago for signaling through G-protein coupled receptors directly activating growth factor receptors such as the EGF receptor (78). This mode of transactivation between receptors seems important for FcRs. Several interesting interactions have been published.

FcRs and Other FcRs

Most of the data regarding transactivation of FcRs to other FcRs is indirect. Nevertheless, several lines of evidence show that co-crosslinking of different FcRs leads to differences in signaling. Vossebeld et al. showed that co-crosslinking FcγRII and FcγRIII lead to more mobilization of intracellular free Ca^{2+}

(29). This study also implied a function for FcγRIII as this PI-linked protein was still able to modulate signaling through FcγRIIA. Other studies have shown that cross-linked FcRs lead to differences in the activation of the MAPkinase signaling pathways (20, 79). Interestingly, co-crosslinking of FcRs leads to differential of adhesive phenotypes dependent on the type of FcR and their polymorphisms (80). This mechanism might be important in the fine tuning of responses of leukocytes with different immune complexes. A next level of complexity comes from studies showing functional antagonistic behavior of FcγRIIA and FcγRIIIB (81). These authors provided evidence that immune complexes that are endocytosed by FcγRIIIB are cleared that is considered as anti-inflammatory whilst this process mediated by FcγRIIA leads to Netosis that is considered to be pro-inflammatory. These studies imply that subtle changes brought about by inside out signaling determines the type of the immune response.

FcRs and Integrins

Most data on FcR signaling in *trans* is through integrins. Many studies imply that FcRs pair with different integrins upon activation with immune complexes or by crosslinking of the receptors by anti-receptor antibodies. However, these experiments in primary cells that cannot be genetically manipulated are difficult to interpret in terms of receptor specific signaling as there will be interplay between these receptors, and other modulating membrane receptors where it is basically impossible to determine which signal originates from which signaling chain. To circumvent these "chicken and the egg" issues experiments have been performed in cell lines ectopically expressing FcRs and integrins. Poo et al. have described the physical interaction between FcγRIII and Mac-1 (CD11b) in fibroblasts (82). A similar finding described the interaction between FcγRII and Mac-1 (57). This latter interaction is important for FcγRII mediated phagocytosis. Indirect experiments show that these interactions are also important in the response of neutrophils with opsonized particles (83). The concept that Mac-1 can transduce signals for other Mac-1 binding partners has been described before (84).

The Interaction Between FcRs and G-Protein Coupled Receptors (GPCR)

The interaction between FcRs and G-protein coupled receptors (GPCR) can cross regulate their functions. It has been established that the function of FcγRII on eosinophils is upregulated by priming evoked by agonists of GPCR (67). However, it is uncertain whether a physical interaction between FcγRII and GPCR is necessary or that the activated GPCR activates the receptor by cytosolic signaling. Relevant is, however, that periplakin that regulates the functionality of FcγRI (CD64) can also bind GPCR's (70) supporting a potential bridging role of periplakin between FcRs and GPCR's. Such functions have been amply described in the control of integrins, which has been recently reviewed by Ye et al. (38).

FcRs With Other Proteins

FcRs with other proteins have been described but one should be aware of the fact that the intimate interaction between integrins and FcRs might preclude the identification of other binding partners: in multimolecular complexes these proteins such as integrin associated protein (85) or thrombospondin (86) might bind to integrins rather than the associated FcR.

INSIDE-OUT CONTROL, MEMBRANE DOMAINS, AND LATERAL MOVEMENT

Up to now the functionality of FcRs has been described as if the receptors are free flowing in the plane of the plasma membrane. This is, however, a too simple view as the membrane is organized in domains with different fluidities. Best studied are the micro domains rich in cholesterol also referred to as lipid rafts (87). But other specialized domains such as found in the lamellipodium (88) and uropods (89) are also characterized as being enriched in important receptors and signaling molecules. Receptors can therefore be localized at different membrane compartments that are relatively slowly interacting. Not much is known regarding the distribution of FcRs in these different domains, but recent studies support the importance of lateral mobility of FcRs in the plain of the membrane and the importance of co-localization in these domains (54). In addition, Ten Broeke et al. provided evidence that dephosphorylation of FcαRI and functional activation of the receptor is associated with enhanced lateral movement of the receptor and possibly an increase in valency of the receptor (43). Moreover, data of Yang et al. implied that cross-linking of FcγRIIIb (CD16b) leads to lipid raft mediated activation of SHP2 (51).

INSIDE-OUT CONTROL AND THE HIGH AFFINITY RECEPTOR FOR IGE, FCεRI

The main emphasis in this review was inside-out control of IgG and IgA receptors as this process was best described in this context. However, several studies clearly indicate that also the function of FcεRI is controlled by inside-out signals. This control has been excellently reviewed by Kraft and Kinet (90). Important for this review is the requirement of expression of the tetraspanin CD63 for optimal functionality of FcεRI on mast cells (91). As CD63 is expressed in granules this finding links degranulation with an optimal function of FcεRI. Several other processes are involved in the control of FcεRI by either activating (92) or inhibiting the receptor (93). These processes are now seen as therapeutic targets in allergic diseases (6).

THE IMPLICATIONS OF INSIDE-OUT CONTROL IN CLINICAL APPLICATIONS OF HUMANIZED ANTIBODIES

The implications of FcR inside-out control for the treatment of patients with clinical humanized antibodies are just emerging. The approach will obviously depend on the requirement of

effector cells in such therapy and the FcR that they express. Treatment with blocking antibodies directed against single molecules (such as cytokines, complement fragments, and chemokines) might not be directly affected by inside-out control of FcRs as these receptors do not have an obvious role here. However, FcRs play a role in clearance of these target-antibody complexes as the majority is cleared by endocytosis and will subsequently be degraded in the lysosomal compartment (53). This may indicate that therapeutic antibodies might be more rapidly cleared in patients with inflammatory diseases that are characterized by the presence of priming mediators in the peripheral blood or tissue (94). Under these conditions inhibition of inside-out control might be a therapeutic target as it might preserve therapeutic doses of these antibodies allowing lower dosing of the antibodies.

The situation with several antibodies might be more complex. Particularly, those antibodies blocking the function of cellular receptors are of interest. On the one hand, one might want to inhibit inside-out control for preservation of sufficient therapeutical concentrations (see above) on the other inside-out activation might be beneficial for the clinical effect. The idea behind this conception is the following. Anti-receptor antibodies or antibodies directed against cell bound cytokines not only block these molecules, but they might also enable the cell expressing these proteins to be killed (95). This is mediated by antibody or complement dependent cytotoxicity: ADCC or CDC, respectively. Binding of antibodies and/or complement to cells leads to opsonization. Phagocytic receptors are particularly directed against multivalent ligands such as a surface covered with antibodies or complement. The phagocytes will then activate the same armamentarium normally employed for the killing of micro-organisms. The result is a cytotoxic response toward the opsonized cell instead of micro-organism. As both complement receptors such as complement receptor 3 (CR3/Mac-1/CD11b) and FcRs such as FcγRIIA (CD32) and FcαRI (CD89) are very sensitive for inside-out activation it will be clear that this activation is very important for the clinical action (53, 84). Not much is known regarding these issues in humans *in vivo* some studies now imply that ADCC is often important for the clinical effect of therapeutic antibodies (96, 97). A clear example is the anti-IL5R antibody, Benralizumab, which functions through ADCC (95) of IL5Rα+ cells [eosinophils, basophils and possibly ILC2 (98)]. The concept of inside-out activation of the ADCC under these conditions has not been applied to these clinical studies.

The overall conclusion whether or not inside-out control should be considered in augmenting the therapeutic is likely to be dependent on the mode(s) of action of the therapeutic antibodies. It is, however, clear that this complexity should be considered gaining optimal therapeutic effectiveness of current and new antibodies.

CONCLUSION

Inside-out control of FcRs as well as integrins functions as a safety lock preventing collateral damage evoked by innate immune effector cells. Here a clear cross-talk is present between the adaptive immune response producing priming mediators and the innate immune system that adapt to these signals. Part of the priming mediators liberated during inflammation leads to inside-out control of FcRs potentiating these receptors. This very complex mechanism is based on modulation of valency of the receptors, their affinity, their interaction with other signaling chains and receptors and their localization in specialized membrane areas such as lipid rafts. The many levels of control will make it possible to fine tune the inside out control with therapeutic molecules only affecting part of this process. This will allow stratified therapy such that the therapeutic effect is maximal while the normal function of phagocytes is preserved.

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The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Role of the IgM Fc Receptor in Immunity and Tolerance

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Immunoglobulin (Ig) M is the first antibody isotype to appear during evolution, ontogeny and immune responses. IgM not only serves as the first line of host defense against infections but also plays an important role in immune regulation and immunological tolerance. For many years, IgM is thought to function by binding to antigen and activating complement system. With the discovery of the IgM Fc receptor (FcμR), it is now clear that IgM can also elicit its function through FcμR. In this review, we will describe the molecular characteristics of FcμR, its role in B cell development, maturation and activation, humoral immune responses, host defense, and immunological tolerance. We will also discuss the functional relationship between IgM-complement and IgM-FcμR pathways in regulating immunity and tolerance. Finally, we will discuss the potential involvement of FcμR in human diseases.

Keywords: IgM, FcμR, BCR signal, humoral immune response, complement

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INTRODUCTION

B cells produce different classes of antibodies (Ab), including IgM, IgD, IgG, IgA, and IgE. Ab constitutes a variable F(ab) region that binds to antigen (Ag) and a constant Fc region that mediates effector function. Cellular receptors for the Fc region mediate a variety of functions including phagocytosis of Ab-opsonized pathogens and induction of cellular cytotoxicity. Recent studies have unveiled three Fc receptors for IgM, including Fcα/μ receptor (Fcα/μR), polymeric immunoglobulin receptor (pIgR), and Fcμ receptor (FcμR). Fcα/μR, pIgR, and FcμR are all type I transmembrane proteins belonging to the immunoglobulin (Ig) gene superfamily. Fcα/μR is expressed by both hematopoietic and non-hematopoietic cells (1, 2), and has been shown to play an important role in humoral immune responses, especially in pro-inflammatory functions of marginal zone B cells in sepsis (3). pIgR is expressed on the basolateral surface of ciliated epithelial cell in the mucosal epithelium (4, 5), but not in hematopoietic cells (6). The main function of pIgR is to transport dimeric IgA and polymeric IgM from the lamina propria across the epithelial barrier to mucosal surfaces (7). FcμR was discovered relatively recently and its function has not been fully elucidated. Here we summarize the results of FcμR published over the past several years, and discuss how it contributes to immunity and tolerance.

MOLECULAR CHARACTERISTICS OF FCμR

The existence of a receptor for IgM was noted more than 40 years ago (8–16). Biochemical analysis revealed that human FcμR had a molecular weight of ∼60-kDa (17). Molecular cloning of *FCMR*, the gene encoding human FcμR, revealed that it is a single copy gene located on chromosome 1q32.2, adjacent to two other IgM associated Fc receptor genes, polymeric Ig receptor gene (*PIGR*) and the gene of FcR for IgA and IgM (*FCAMR*) (18). Human FcμR is a type I transmembrane protein of 390 amino acids (aa), composed of a 234-aa extracellular domain, a 21-aa transmembrane

segment, and a 118-aa cytoplasmic tail (19, 20). BW5147 T cells ectopically expressing human FcμR exhibited specific binding to IgM but not any other Ab isotypes, demonstrating that FcμR is the bona-fide receptor for IgM (18). Unlike many other FcRs, the cytoplasmic tail of human FcμR does not contain any immunoreceptor tyrosine-based activation (ITAM) or inhibitory (ITIM) motifs. Instead, it contains conserved serine and tyrosine residues, which match the recently described Ig-tail tyrosine (ITT) motif (21, 22). Crosslinking human FcμR with either anti-FcμR monoclonal antibodies or preformed IgM immune complexes triggered the phosphorylation of these serine and tyrosine residues in FcμR-overexpressing BW5147 T cells, suggesting that FcμR could serve as an ITT phosphorylation molecule to interact with and influence the B cell receptor (BCR) signaling (23). Human FcμR is predominantly expressed by B, T, and NK cells, but not by monocytes, granulocytes, erythrocytes, and platelets (18). Human FcμR binds more efficiently to the Fc portion of IgM reactive with surface proteins than to the Fc portion of free IgM (24), suggesting that FcμR might modulate the signal of B, T, and NK cell surface receptors or proteins recognized by natural or immune IgM.

The mouse FcμR gene (*Fcmr*) is also a single copy gene located on chromosome 1 (56.89 cM), adjacent to *Pigr* and *Fcamr* (25). Although mouse and human FcμR have similar molecular structure, they share only 54% aa identity. Mouse FcμR also specifically binds to IgM (25, 26). Unlike human FcμR, we found that mouse FcμR is predominantly expressed in B lymphocytes by both microarray of a panel of immune cell types and FACS analyses (25, 27, 28). However, others have reported that monocytes, macrophages, granulocytes, and dendritic cells also express FcμR (29, 30). The expression levels of Mouse FcμR are different among different B cell subsets. The hierarchy of FcμR levels on various B cell subsets is as follows: marginal zone precursor (MZP, $IgM^{hi}CD21^{hi}CD23^{hi}$) > follicular B (FOB, $IgM^{lo}CD21^{lo}CD23^{hi}$) > marginal zone B (MZB, $IgM^{hi}CD21^{hi}CD23^{lo}$) > newly formed B ($CD93^{+}CD21^{-}CD23^{-}$) cells (28, 31). FcμR expression level is indistinguishable between B1 ($CD5^{+}$) and B2 ($CD5^{-}$) cells in the spleen. In the peritoneal cavity, FcμR expression level in each B cell subsets follows the order: B2 ($CD11b^{-}CD5^{-}$) ≅ B1a ($CD5^{+}$) > B1b ($CD11b^{+}CD5^{-}$) cells (31, 32). In addition, FcμR expression is very low in pro-B ($B220^{+}CD43^{+}$) and pre-B ($B220^{+}CD43^{-}IgM^{-}$) cells, and slightly upregulated in immature B cells ($B220^{dull}IgM^{+}$) in the bone marrow (BM) (27, 31, 33). FcμR expression in the germinal center (GC) B cells ($CD95^{+}GL7^{+}$) is much lower than that in naïve B cells (27), suggesting that FcμR is down-modulated during GC reaction. FcμR is expressed at higher levels in plasmablasts compared to plasma cells. Intriguingly, FcμR is also expressed by IgG- or IgA-positive B cells, suggesting that it may play a role in switched B cells (32).

It is intriguing that genes encoding FcμR, Fcα/μR, and pIgR are located in the same chromosomal region (18, 25), suggesting that these genes are evolutionarily related and might have derived from a common ancestor gene. However, in contrast to FcμR which only binds to IgM, Fcα/μR binds both IgM and IgA (3, 34, 35). Moreover, pIgR binds both IgM and IgA via their associated J chains and is essential for the transcytosis of polymeric IgA and IgM to the gut (36). The expression pattern is also quite different among these receptors. FcμR is predominantly expressed by B cells in mice and by B, T, and NK cells in humans (18, 25). In contrast, Fcα/μR is expressed by macrophages, B cells, intestinal lamina propria and several other cell types (35), and pIgR is mainly expressed on the intestinal epithelial cells (4, 5). Although FcμR was originally designated as Fas apoptotic inhibitory molecule 3 or TOSO (37), it is now clear that both human and mouse FcμR have no inhibitory activity against Fas-mediated apoptosis (38, 39).

FCμR IN B CELL DEVELOPMENT AND MATURATION

Several *Fcmr*-deficient (KO) and B-cell-specific deletion of *Fcmr* (BKO) mouse strains have been generated. (1) We and Kubagawa et al. share the constitutive FcμR knockout strain ($Fcmr^{tm1Ohno}$) in which exons 2–4 were deleted in 129/Sv ES cells and the mutant mice had been backcrossed to C57BL/6 mice for > 12 generations. The neo gene used in drug selection was removed by crossing with Cre-Tg mice (27, 28, 32, 40–42); (2) Mak et al. and Coligan et al. share the constitutive FcμR knockout strain ($Fcmr^{tm1Mak}$) where exons 2–8 were deleted in 129/Sv ES cells and the mutant mice had been backcrossed to C57BL/6 mice. The neo gene remained in the targeted allele (29–31, 43, 44); (3) Lee et al. have the constitutive FcμR knockout strain ($Fcmr^{tm1.2Khl}$) and a strain with floxed *Fcmr* allele, with exons 4–7 were deleted or flanked by loxP sites, respectively. No neo gene remained in the targeted allele and both mice are on a pure B6 background (45–47); Baumgarth et al. generated the $Fcmr^{flx/flx}Cd19\text{-}Cre^{+}$ strain in which exon 4 was deleted by CD19-driven Cre. The mutant mice are on a pure B6 background (33, 48). A comparison of the phenotypes of $Fcmr^{-/-}$ mice generated and/or analyzed by different groups is shown in **Table 1**.

B cell development proceeds from pro-B, pre-B to immature B cells in BM (49). Immature B cells then migrate to the periphery where they further differentiate into various mature B cell subsets that play distinct roles. The survival and maturation of B cells are dependent on the strength of tonic BCR signal (50, 51). Studies from our group, Honjo et al. and Nguyen et al. revealed that FcμR deficiency did not significantly affect B cell development, but altered the numbers of different B cell subsets (32, 33). We and Honjo et al. found that MZB were severely reduced in KO mice (27, 32) whereas Nguyen et al. found decreased proportion of MZB but the absolute numbers of MZB were not affected (**Table 1**) (33). Honjo et al., Choi et al., and Nguyen et al. reported that the splenic B1 cells were increased in KO mice (31–33). More recently, we found reduced tonic BCR signaling in FcμR-deficient MZB, which we think led to their decreased numbers in KO mice (28). In contrast, Honjo et al. suggested that the reduction of MZB in KO mice was due to their rapid differentiation into plasma cells (41). Lee et al. found decreased numbers of B cells in the spleen and lymph nodes (47). Choi et al. found that B-1a were increased but B-2 were decreased in the peritoneal cavity and that FOB were decreased in the

TABLE 1 | Comparison of the phenotypes of *Fcmr*$^{-/-}$ mice generated/analyzed by different groups.

Mouse strain		***Fcmr*^{tm1Ohno}**		***Fcmr*^{tm1Mak}**		***Fcmr*$^{tm1.2Khl}$**	***Fcmr*$^{flx/flx}$ CD19-Cre+**
Targeting strategy		Exons 2–4 were deleted in 129/Sv ES cells and the mice backcrossed to C57BL/6 mice. The neo gene was removed		Exons 2–8 were deleted in 129/Sv ES cells and the mice backcrossed to C57BL/6 mice. The neo gene was not removed		Constitutive FcμR knockout strain and a conditional knockout with exons 4–7 deleted. Pure B6 background	Exon 4 was deleted by CD19-driven Cre. Pure B6 background
Research group		Hiromi Kubagawa	Ji-Yang Wang	John E. Coligan	Tak W. Mak	Kyeong-Hee Lee	Nicole Baumgarth
Related references		(32, 41)	(27, 28, 40)	(31)	(29, 30, 44)	(45, 47)	(33, 48)
B & T cells	BM	Pro-B, Pre-B, Immature B, Recirculating B	Pro-B, Pre-B, Immature B, Recirculating B	Pro-B, Pre-B, Immature B, Recirculating B			Pro-B, Pre-B, Immature B, Recirculating B, B1
	Spleen	Total T, Total B, FOB, Newly formed B, Regulatory B, MZB, B1	Total T, Total B, FOB, T3, MZB	Total B, Newly formed B, Regulatory B, MZB, B1, FOB		Total T, Mature B, Newly formed B, Regulatory B, MZB, B1a,	Total B, FOB number, Newly formed B, MZB number, MZB ratio, B1, B1a, GCB
	PC	Total T, Total B, B1a, B1b, B2	B1a	Total B, B1a, B1b, B2		B1a, B1b	
B cell function			BCR-triggered Ca^{2+} influx, antigen presentation, CSR, B cell survival induced by BCR cross-linking, BCR-triggered activation of non-canonical NF-κB pathway	B cell survival induced by BCR cross-linking		B cell activation	Turnover and survival of B cells
Homeostasis & Humoral immune responses	Basal Ig levels	IgG2b, IgG2c, IgA, IgM, IgG3	IgG3, IgG2b IgG2c, IgA, IgM	3 month old: IgM, IgG3, IgG2b IgG2a, IgA, IgG1; 6 month old: IgM, IgG1, IgG2b IgG2a, IgG3, IgA			IgG, IgA, IgM
	TI response	Phosphorylcholine response	TI-1 & TI-2 responses, MZB response to LPS	GCB & PC	Response to LPS		
	TD response	Affinity maturation of Abs, primary IgG1 and secondary IgM anti-CGG responses	GC formation, Memory B and plasma cell, Ab production in primary and secondary responses	GCB, PC, IgM, IgG2a			
Infectious immunity		Low dose of R36A: increased IgM and IgG3 responses; High dose of R36A: no increase	*C. rodentium*-induced sepsis		*Listeria*-induced & persistence-prone infection	TNFα-mediated liver damage, Influenza virus infection	Influenza virus infection
B cell tolerance		IgM and IgG anti-dsDNA, ANAs; Serum auto-antibody titers and Mott cell formation in FcμR KO B6/lpr mice but no lupus-like nephritis	IgG anti-dsDNA Abs, rheumatoid factor, ANAs	IgG anti-dsDNA and ANAs	EAE	Differentiation/maintenance of regulatory B cells, IgM an IgG anti-dsDNA, or anti-ssDNA	IgM and IgG anti-dsDNA

Black: not affected.
Blue: increased or enhanced.
Red: decreased or impaired.
Blank: not investigated.

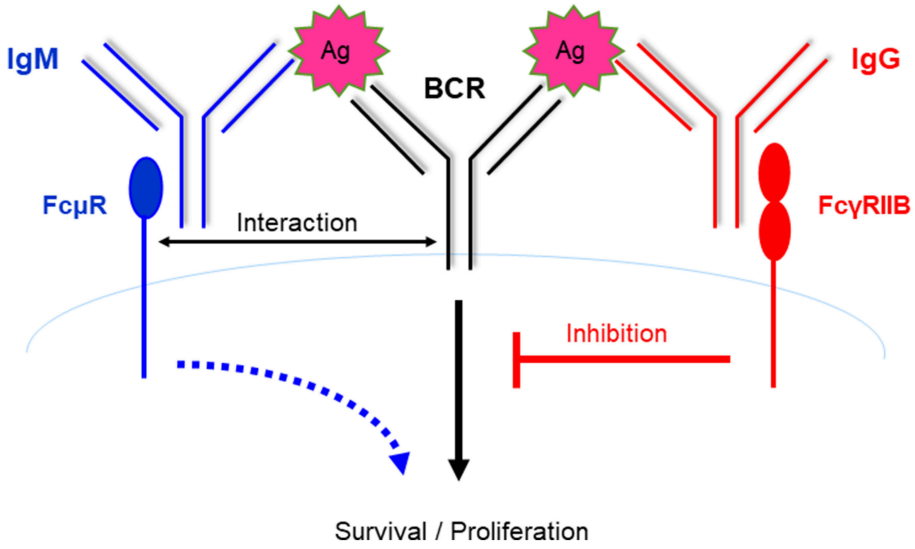

FIGURE 1 | B cells express two types of Fc receptors of opposing functions. FcμR promotes B cell activation via interacting with the BCR and potentiating BCR signaling. In contrast, B cells express FcγRIIB, which inhibits B cell activation upon binding to immune complexes containing IgG and the cognate Ag, which then results in colligation of FcγRIIB and the BCR. Therefore, B cells express two types of Fc receptors of opposing functions. It is suggested that these two receptors function in a spatial-temporal manner to positively and negatively regulate B cell activation during humoral immune responses (see **Figure 2**).

spleen (**Table 1**) (31), which were similar to the phenotypes found in $S\mu^{-/-}$ mice that lack secreted IgM (52, 53). Taken together, these results indicate that FcμR affects the maturation or differentiation of various B cell subsets.

FCμR IN B CELL SURVIVAL AND ACTIVATION

We found that FcμR cell surface expression was upregulated after BCR cross-linking with anti-IgM Abs but only moderately increased by CD40L or LPS stimulation under *in vitro* culture conditions (40). Choi et al. reported that FcμR transcript levels were markedly reduced by stimulation of spleen B cells with anti-IgM, LPS or anti-CD40 (31), suggesting that FcμR expression is regulated at both transcriptional and posttranscriptional levels. Moreover, we and others demonstrated that FcμR specifically enhanced B cell survival induced by anti-IgM stimulation (**Table 1**) (27, 31, 40). Immunofluorescence and co-immunoprecipitation revealed physical interaction between FcμR and BCR on the plasma membrane of primary B cells (40). Although FcμR deficient B cells exhibited normal Ca^{2+} influx after BCR crosslinking, their survival was reduced compared with WT B cells (27), indicating that FcμR did not affect the early BCR signaling event such as Ca^{2+} influx but affected the late response such as B cell survival. Analysis of signaling molecules downstream of BCR revealed that FcμR promoted the activation of the non-canonical NF-κB pathway and the induction of BCL-xL (40). These results suggest that FcμR and BCR cooperate in signal transduction to promote B cell survival. FcμR does not contain any ITAM motifs but instead contains several conserved tyrosine and serine residues in its cytoplasmic tail (19, 20, 23, 26). A detailed mutational analysis has revealed that the tyrosines 315, 366, and 385 are not required for ligand (IgM) binding. However, tyrosine 315, as well as the entire intracellular domain, was shown to be required for inhibiting an IgM anti-FAS Ab-induced apoptosis (24). It remains to be investigated how FcμR specifically affects the late phase of BCR signaling and whether these tyrosine and serine residues are involved.

It is well-known that B cells express FcγRIIB, which inhibits BCR signaling and B cell activation upon binding IgG-Ag immune complexes, which then results in colligation of FcγRIIB and the BCR. Therefore, B cells express two types of Fc receptors, FcμR and FcγRIIB, which promotes and inhibits BCR signaling and B cell activation, respectively (**Figure 1**). More recently, Nguyen et al. reported that FcμR limited tonic BCR signaling in immature B cells by regulating the expression of IgM BCR (33). Therefore, FcμR regulates both the cell surface expression and the function of BCR.

ROLE OF FCμR IN HUMORAL IMMUNE RESPONSES

The basal Ig levels reflect the immune homeostasis at the steady state. We found that basal serum IgM levels were elevated in the absence of FcμR in a gene dosage-dependent manner, suggesting that a portion of the serum IgM actually binds to the FcμR in WT mice (27). Nguyen et al. found the same results and attributed the high IgM level to the elevated numbers and hyper-activation of B1 cells in the spleen (33). In addition, Honjo et al. found that IgM levels were elevated and that the IgG3 levels were slightly elevated in KO mice (32). In contrast, Choi et al. reported that only IgG1 levels were reduced in 3-month old mice and IgG3 and IgA levels were slightly elevated in 6-month old mice (31). Therefore, FcμR-deficient mice generated by different groups all exhibited increased levels of serum IgM and/or IgG3 (**Table 1**). These results implicate a role for FcμR in B cell homeostasis.

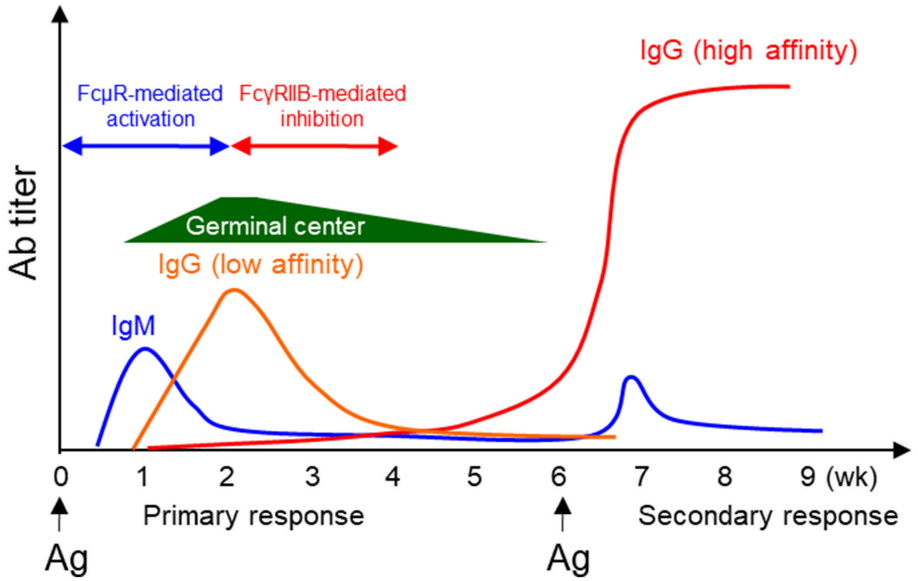

FIGURE 2 | Positive and negative regulation of humoral immune responses by FcμR and FcγRIIB. During a typical T-D humoral immune response, Ag-specific IgM is produced first, followed by IgG production. Based on the results that FcμR promotes B cell activation and Ab production and the earlier findings that FcγRIIB inhibits B cell activation and Ab production, we propose an autoregulatory mechanism for T-D humoral immune responses. During the early phase of the response, B cell activation is enhanced by FcμR-mediated positive signals. However, at a later phase of the response, further B cell activation is suppressed by FcγRIIB-mediated inhibitory signal.

We found that KO mice had significantly decreased production of NP-specific IgG1 during both primary and secondary responses against a T-dependent (T-D) Ag, NP-CGG (27, 28), likely due to impaired GC formation and reduced memory and plasma cell differentiation. Similarly, Honjo et al. found impaired primary IgG1 and secondary IgM anti-CGG responses, but normal Ab affinity maturation (32). During humoral immune responses to T-D Ag, Ag-specific IgM is first produced, which is followed by the production of Ag-specific IgG. Based on our results that FcμR is required for efficient Ab production and the earlier findings that FcγRIIB inhibits B cell activation and Ab production, we propose an autoregulatory mechanism for T-D humoral immune responses [(27) and **Figure 2**]. During the early phase of the response, when the amount of Ag-specific IgM is greater than that of Ag-specific IgG, B cell activation is enhanced by FcμR-mediated positive signals. However, during the later phase of the response, when the amount of Ag-specific IgG is greater than that of Ag-specific IgM, further B cell activation is suppressed by FcγRIIB-mediated inhibitory signal (**Figure 2**). B cell activation and Ab production can thus be positively and negatively regulated by Ag-specific IgM and IgG present in the local environment, respectively.

Consistent with the reduced survival in FcμR-deficient B cells after BCR crosslinking, FcμR KO mice had decreased Ab production against a type 2 T-independent (T-I) Ag, NP-FICOLL (27), since response to this type of Ag is largely dependent on BCR signal. Additionally, we found that FcμR KO mice had impaired Ab production against a type 1 T-I Ag, NP-LPS (28), which activates B cells through both BCR and toll-like receptor 4. Moreover, we found that MZB in KO were not activated upon LPS injection (28). Since MZB cells are thought to participate in the response to LPS, the reduced Ab production to NP-LPS immunization could be due to both a reduction in the number of MZB cells and their impaired response to LPS. Our results are consistent with the earlier finding by Lang et al. that FcμR-deficient mice had reduced LPS response *in vivo* (29). Choi et al. found elevated numbers of GC B cells and accelerated plasma cell formation during type 1 and 2 T-I immune responses and secondary T-D immune responses (31). In addition, the plasma cell formation in primary T-D immune response was also increased (summarized in **Table 1**). The reason for the discrepancies among results from different groups is unclear but could in part be attributable to the differences in the targeting strategy, the immunization protocol, and the genetic background as well as rearing environment of these mutant mice. Collectively, these results suggest that FcμR regulates humoral immune responses.

FcμR IN INFECTIOUS IMMUNITY

As summarized in **Table 1**, FcμR-deficient mice generated a higher titer of anti-phosphorylcholine Ab and a lower titer of anti-protein Ab than did WT mice when infected with a low dose of live non-encapsulated strain of *Streptococcus pneumoniae* (R36A) (32). However, a high dose of pathogen infection induced no significant difference in Ab production between WT and KO mice. We found that FcμR protected mice against sepsis induced by *Citrobacter rodentium*, a gram-negative bacterium that has LPS on the outer membrane (28). Similarly, Lang et al. found that the absence of FcμR resulted in limited cytokine production after

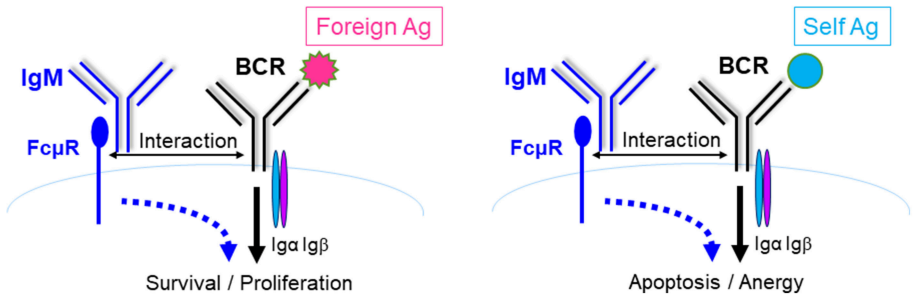

FIGURE 3 | A model for FcμR-mediated immunity and tolerance. FcμR promotes the survival and activation of mature B cells by interacting with the BCR and potentiating foreign Ag-triggered BCR signaling **(left)**. By analogy, FcμR might also promote self Ag-triggered BCR signaling in immature B cells and contribute to the deletion/anergy of autoreactive immature B cells in the BM **(right)**. Ag-specific IgM/IgG are illustrated in the scheme shown in **Figures 1**, **2** and **4** to suggest that those reactions occur during an immune response. In contrast, IgM shown in this scheme is not Ag-specific to implicate that these reactions can occur in the absence of Ag-specific IgM.

Listeria monocytogenes (a gram-positive bacterium) infection and increased death of the infected KO mice (29). They also found that FcμR was required for the control of persistence-prone virus infection in a lymphocytic choriomeningitis virus model system (44). In addition, Yu et al. reported that FcμR deficiency resulted in increased numbers of IL-10–producing B cells, which mediated regulation of T cell immunity during influenza infection (45). On the contrary, Nguyen et al. found that FcμR expression on B cells, but not Fcα/uR expression or complement activation, was important for the antiviral IgG responses (48). B cell-specific KO mice lacked robust clonal expansion of influenza hemagglutinin-specific B cells early after infection and developed fewer IgG plasma cells and memory B cells in the spleen and BM, compared with WT mice (48). These results suggest that FcμR has important roles in B cell responses to protein and non-protein determinants of live pathogens and in cooperating with other immune cells to protect the mice against infection.

FCμR IN B CELL TOLERANCE

B cell central tolerance ensures autoreactive immature B cells to undergo clonal deletion, anergy or receptor editing while peripheral tolerance functions to delete autoreactive B cells generated during GC reaction. We and others found that KO generated autoreactive antibodies including anti-dsDNA, rheumatoid factor, and anti-nuclear antibodies (27, 32, 33, 41, 45). Honjo et al. crossed FcμR-deficient mice with the Fas-deficient autoimmune-prone B6.MRL *Fas*$^{lpr/lpr}$ mice (B6/lpr), and found that the double mutant mice had accelerated development of autoreactive Ab including anti-dsDNA and anti-Sm Ab (41). They also found enhanced formation of Mott cells, aberrant plasma cells which accumulate large amount of Ig in the rough endoplasmic reticulum, in KO mice. Nevertheless, KO mice with autoimmune-prone background have normal kidney function and equal mortality compared to control group (41). Brenner et al. reported that KO mice were protected from the development of severe experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis. Their results suggested that FcμR regulated the function of dendritic and regulatory T cells (30). Collectively, a common feature of KO and BKO generated by different groups is the production of various autoantibodies (**Table 1**). It remains to be investigated how FcμR regulates B cell tolerance. We have shown that FcμR promotes B cell survival and activation by interacting with BCR and potentiating Ag-triggered BCR signaling (**Figure 3**, left panel). By analogy, we think that FcμR might also promote self Ag-triggered BCR signaling in immature B cells and contribute to the deletion/anergy of autoreactive immature B cells in the BM (**Figure 3**, right panel). Further studies are required to clarify whether and how FcμR contributes to B cell central or peripheral tolerance.

FUNCTIONAL RELATIONSHIP BETWEEN IgM-COMPLEMENT AND IgM-FCμR PATHWAYS

IgM is the first Ab to appear during evolution and the only isotype produced by all species of jawed vertebrates (54–56). It is also the first isotype produced during a T-D immune response and is the first line of host defense (57). IgM is not only an effector molecule, but also regulates humoral immune response. Earlier studies suggested that IgM promotes the production of antigen-specific IgG via activating complement. However, a recent study by Heyman's group demonstrated that mice expressing a mutant IgM unable to activate complement (Cμ13) had completely normal humoral immune responses (58), thus raising the possibly that in addition to complement activation, there are alternative pathways by which IgM elicits its function. As discussed above, IgM can elicit its function through FcμR. Therefore, both IgM-FcμR and IgM-complement pathways function to regulate B cell survival and activation (**Figure 4**). It remains to be investigated whether these two pathways function cooperatively, independently, or competitively.

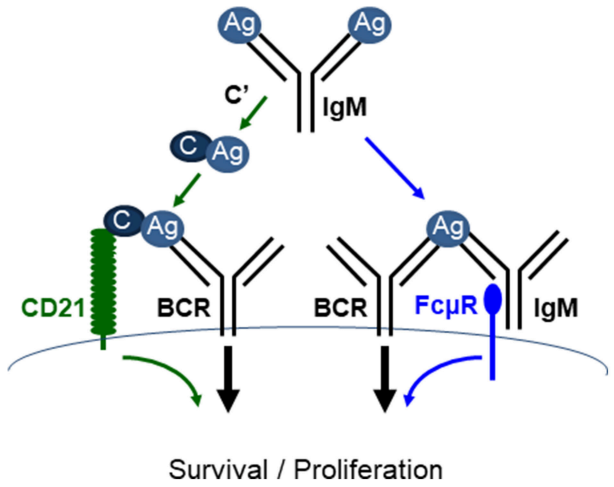

FIGURE 4 | FcμR and complement receptor promote mature B cell survival and activation. IgM (pentamer; for simplicity a monomeric IgM is depicted) binds to antigens (Ag) and the resulting IgM-Ag complexes can enhance B cell survival and activation through at least two pathways. (1) IgM-Ag complexes can activate complement (C') cascade, resulting in C' fixation on the Ag, which can crosslink BCR and C' receptor (CD21) on B cells (green arrows). (2) IgM-Ag complexes can crosslink BCR and FcμR on B cells (blue arrows). It remains to be elucidated whether these two pathways function cooperatively, independently or competitively [adapted from Ouchida et al. (27)].

FCμR IN HUMAN DISEASES

Human FcμR was shown to be overexpressed and associated with the anti-apoptotic characteristic in chronic lymphocytic leukemia (CLL) (59, 60). CLL is a malignancy of mature IgM^+ B cells that exhibit features of polyreactive, partially anergized B cells related to memory B cells (60). Several studies showed that *FCMR* expression in CLL was significantly higher than that in healthy controls and other B cell lymphoproliferative diseases (59, 61–63). In addition, CLL patients also had higher serum titers of FcμR compared with healthy donors. The serum FcμR, a 40-kDa soluble form of the receptor generated by alternative splicing, was produced by both CLL B and non-CLL B cells (64). Cox regression analysis indicated that high expression of *FCMR* was an independent indicator for shorter treatment-free survival in CLL (64). Thus, FcμR is associated with the disease progression and patient survival and may serve as a prognostic factor. Interestingly, FcμR can even be used as a target for a more selective treatment of CLL by T cells expressing a chimeric antigen receptor (CAR-T), and initial studies have implicated a superior therapeutic index with anti-FcμR CAR-T cells for the treatment of CLL compared with the currently used therapies (65).

The reason that causes FcμR upregulation in CLL remains unclear. A negative correlation was observed between age and FcμR expression (59). In addition, overexpression of FCMR seemed to promote the chromosomal abnormalities (61). These shreds of evidence suggest that FcμR expression is related to the degree of genomic activity. Intriguingly, surface FcμR levels were also significantly elevated in the non-CLL B cells and T cells, suggesting that abnormal expression of FcμR is associated with systemic gene regulation (64). FcμR expression is significantly upregulated by BCR stimulation but decreased by CD40 ligation, which suggested that autoreactive BCR signaling as a key mediator of apoptosis resistance in CLL (63). Besides, FcμR expression on CLL cells is downmodulated at both the mRNA and protein levels by TLR7 and TLR9 agonists (60). This study also revealed that FcμR not only localized to the cell membrane but also accumulated in the trans-Golgi network (60). FcμR may internalize IgM-Ag complexes and thus serve as a receptor for the delivery of therapeutic Ab–drug conjugates into CLL cells (60). In addition, based on the findings in mice, human FcμR may have some roles in TNFα-mediated liver damage (47), malaria vaccine promotion (46), and the function of pancreatic islets (66).

CONCLUSION

IgM is an old immunoglobulin isotype, which can bind to Ag with high avidity and activate the complement cascade. Its authentic and specific Fc receptor (FcμR) is the last one to be explored after Fcα/μR and pIgR. Although there are some discrepancies regarding the function of FcμR published by different groups, the following common abnormal phenotypes have been observed: (1) alterations in B cell maturation and differentiation; (2) impaired humoral immune responses; (3) autoantibody production. In addition, FcμR appears to contribute to the initiation/progression of human CLL and has recently been tested as a therapeutic target for treating CLL. Yet still many questions remain to be answered, including the function of FcμR in the generation, maintenance and activation of memory B cells, and in host defense mediated by natural IgM produced by B-1 and Ag-specific IgM produced by B-2 cells. Further studies are required to fully uncover the function of FcμR in immunity and tolerance.

AUTHOR CONTRIBUTIONS

JL provided a draft of the manuscript. YW completed the references. EX and RH provided all the figures. QL revised the manuscript. HO corrected the manuscript. J-YW designed the outline and made the final corrections of the manuscript.

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REVIEW
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IgA and FcαRI: Pathological Roles and Therapeutic Opportunities

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Immunoglobulin A (IgA) is the most abundant antibody class present at mucosal surfaces. The production of IgA exceeds the production of all other antibodies combined, supporting its prominent role in host-pathogen defense. IgA closely interacts with the intestinal microbiota to enhance its diversity, and IgA has a passive protective role via immune exclusion. Additionally, inhibitory ITAMi signaling via the IgA Fc receptor (FcαRI; CD89) by monomeric IgA may play a role in maintaining homeostatic conditions. By contrast, IgA immune complexes (e.g., opsonized pathogens) potently activate immune cells via cross-linking FcαRI, thereby inducing pro-inflammatory responses resulting in elimination of pathogens. The importance of IgA in removal of pathogens is emphasized by the fact that several pathogens developed mechanisms to break down IgA or evade FcαRI-mediated activation of immune cells. Augmented or aberrant presence of IgA immune complexes can result in excessive neutrophil activation, potentially leading to severe tissue damage in multiple inflammatory, or autoimmune diseases. Influencing IgA or FcαRI-mediated functions therefore provides several therapeutic possibilities. On the one hand (passive) IgA vaccination strategies can be developed for protection against infections. Furthermore, IgA monoclonal antibodies that are directed against tumor antigens may be effective as cancer treatment. On the other hand, induction of ITAMi signaling via FcαRI may reduce allergy or inflammation, whereas blocking FcαRI with monoclonal antibodies, or peptides may resolve IgA-induced tissue damage. In this review both (patho)physiological roles as well as therapeutic possibilities of the IgA-FcαRI axis are addressed.

Keywords: IgA, CD89, mucosa, autoimmunity, IgA deficiency, microbiome, vaccination, therapy

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INTRODUCTION

Immunoglobulins have important functions in immunity (1, 2). In mucosal areas like the gastrointestinal, genitourinary, and respiratory tracts, IgA is the predominant antibody present (3). It plays a key role at these surfaces, which are continuously exposed to antigens, food, and (commensal) microorganisms. Keeping a tight balance by tolerating commensals and harmless (food) antigens on the one hand and providing protection against harmful pathogens on the other hand is a challenging role of mucosal immunity. Mucosal IgA protects the host by diversifying the microbiota, neutralizing toxins and viruses, blocking colonization and penetration of pathogenic bacteria, clearing unwanted particles, and promoting sampling of antigens (4). Mucosal IgA is generally considered as a neutralizing, non-activating antibody. In serum, IgA is the second most

abundant antibody after IgG. In contrast to mucosal IgA, the roles of serum IgA are relatively unexplored (5).

IgA deficiency is a relatively common disease with limited effects on human health (6), which supported the notion of IgA as a non-inflammatory antibody. By contrast, increased serum IgA levels or IgA autoantibodies have been reported in multiple (inflammatory) diseases including IgA nephropathy (IgAN), IgA vasculitis, dermatitis herpetiformis, celiac disease, inflammatory bowel disease (IBD), Sjögren's syndrome, ankylosing spondylitis, alcoholic liver cirrhosis, and Acquired immunodeficiency syndrome (7–9), although the role of IgA in pathology is still ill-understood.

IgA can interact with several receptors that are present on a variety of (immune) cells. The polymeric immunoglobulin receptor (pIgR) is present on the basolateral side of epithelial cells and mediates transport of dimeric IgA (dIgA) to the mucosal lumen, where it is released as secretory IgA (SIgA) (10). Retrograde transport of SIgA immune complexes back into the lamina propria can occur via the transferrin receptor (Tfr or CD71) on epithelial cells or through microfold cells (M cells), possible via interaction with Dectin-1 (11, 12). It has been reported that SIgA immune complexes in the lamina propria can be taken up by sub-epithelial dendritic cells (DCs) through interaction with Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (13). B cells were shown to express the inhibitory IgA receptor Fc receptor-like 4 (FcRL4), which was suggested to contribute to the regulation of mucosal IgA responses (14). In the liver, hepatocytes express the asialoglycoprotein receptor (ASGPR) that is involved in clearance of IgA from the circulation (15). Mesangial cells in the glomeruli of the kidneys express CD71 and the recently identified β-1,4-galactosyltransferase as potential receptors to clear IgA (16). Furthermore, an Fc alpha/mu receptor is expressed in gut, spleen and lymph nodes, although its functions remain to be elucidated (17). Finally, the IgA Fc receptor FcαRI (CD89) is expressed by myeloid cells (18). It was demonstrated that interaction of monomeric serum IgA with FcαRI induces inhibitory signals (19, 20). As such, it is thought that IgA has an anti-inflammatory role during homeostatic conditions. By contrast, IgA immune complexes bind avidly to FcαRI, resulting in cross-linking and induction of pro-inflammatory responses, which may play an important role in resolving (mucosal) infections. Additionally, it was suggested that the presence of excessive IgA immune complexes can lead to uncontrolled and disproportionate immune cell activation, which leads to severe tissue damage in autoimmune diseases, such as IgA blistering diseases and rheumatoid arthritis (RA) (18). The number of diverse IgA receptors, their differential expression on cells and the distinct IgA-induced effector functions support the complex roles of this antibody in maintaining homeostatic conditions, which go beyond the dogma of IgA as a non-inflammatory regulator of mucosal immunity. This is further supported by the fact that several bacteria have evolved to escape IgA-mediated functions by for instance producing IgA1 proteases or expressing molecules that hamper interaction with IgA receptors (21–23).

This review addresses the different functions of IgA, its interaction with FcαRI,—as the best characterized IgA receptor -, in health and disease and the possibilities to target either molecule for therapeutic strategies.

Abbreviations: ASGPR, Asialoglycoprotein receptor; BsAb, Bispecific antibodies; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; DCs, Dendritic cells; dIgA, Dimeric IgA; FcαRI, Fc alpha receptor; FcRL4, Fc receptor-like 4; fMLP, N-formylmethionyl-leucyl-phenylalanine; GF, Germ free; GM-CSF, Granulocyte-macrophage colony–stimulating factor, ; IBD, Inflammatory bowel disease; IFN-γ, Interferon-gamma; IgA, Immunoglobulin A; IgAN, IgA nephropathy; IL, Interleukin; ITAM, Immunoreceptor tyrosine-based activation motif; ITAMi, Inhibitory immunoreceptor tyrosine-based activation motif; kDa, Kilodalton; LABD, Linear IgA Bullous disease; LPS, Lipopolysaccharide; LTB4, Leukotriene B4; M cells, Microfold cells; pIgR, Polymeric immunoglobulin receptor; RF, Rheumatoid factor; SC, Secretory component; SIgA, Secretory IgA; SIgM, Secretory IgM; Tfr, Transferrin receptor; TGF-β, Transforming growth factor-beta; TLR, Toll like receptor; TNF-α, Tumor necrosis factor-alpha.

STRUCTURE OF IgA

IgA is the most predominant antibody produced by humans with a synthesis rate of 66 mg/kg each day. IgA can be subdivided into IgA1 and IgA2 (**Figure 1A**). The IgA1 hinge region contains multiple O-linked glycans and two N-linked glycosylation sites per heavy chain. The truncated hinge of IgA2 lacks O-glycans and each heavy chain contains two additional N-glycans (25).

IgA1 comprises 80–85% of total human serum IgA (1–3 mg/ml) and is prevalent on many mucosal surfaces including the nasal, bronchial, gastric, and small intestinal mucosa. IgA2 is predominantly present in the colon (26). This division of IgA1 and IgA2 between the small and large intestine may reflect the luminal distribution of food proteins and gram-negative bacteria, which is supported by findings that bacterial overgrowth in the small intestine shifts production toward IgA2 (27). Whereas, IgA1 is susceptible to proteolytic cleavage due to a longer hinge region, IgA2 is resistant to bacterial proteases that are present in the lumen of mucosal areas (6, 21).

Serum IgA is predominantly monomeric in humans and primates (while dimeric in other animals) and is produced by local plasma cells in the bone marrow, spleen, and lymph nodes. IgA at mucosal surfaces is produced by local plasma cells as dimeric molecules, although small amounts of monomers, trimers and tetramers or polymers can also be present (1, 3). Dimeric IgA is composed of two monomers, which are linked with a J-chain (**Figure 1B**). It associates with the pIgR present at the basolateral side of epithelial cells in mucosal surfaces, after which dIgA is transported across the epithelium and released into the lumen. For every molecule of dIgA that is transported across the epithelium, one pIgR is needed (10, 28, 29). At the luminal side, pIgR is cleaved and a part, referred to as secretory component (SC), remains attached thereby forming SIgA (**Figure 1B**). Secretory component is a hydrophilic and highly (N- and O-linked) glycosylated negatively charged molecule, which protects SIgA from degradation when present in luminal secretions (30). Glycan removal led to reduced binding capacity of SIgA to gram-positive bacteria, indicating the essential role of carbohydrate structures in efficient IgA coating of (mainly commensal) bacteria (31).

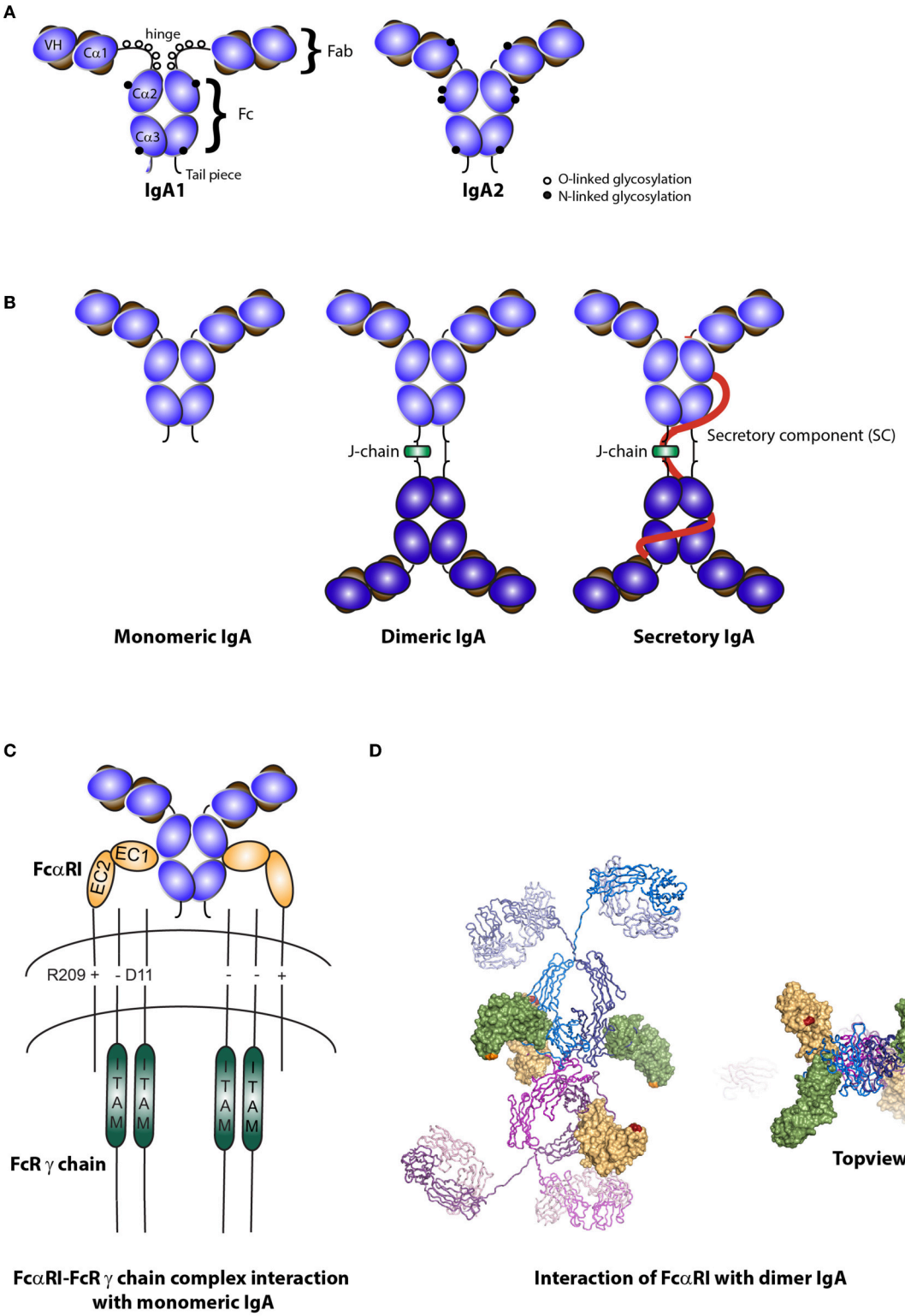

FIGURE 1 | Structure of IgA isotypes and the IgA Fc receptor (FcαRI). **(A)** IgA1 vs. IgA2. IgA consists of two heavy chains (blue), each composed of three constant regions and one variable region, and two light chains (brown) that consist of one constant and one variable region. IgA1 contains a hinge region with O-linked glycosylation. **(B)** Monomeric, dimeric IgA, and secretory IgA. Dimeric IgA consist of two IgA molecules that are linked with a J-chain (green). Secretory IgA contains an additional molecule, the secretory component (SC; red). **(C)** Structure of FcαRI. FcαRI consist of a transmembrane domain, a short cytoplasmic tail and two

(Continued)

FIGURE 1 | extracellular domains (EC1 & EC2). It is associated with the signaling FcR γ chain via an electrostatic interaction. The IgA heavy chain junction of Cα2 and Cα3 binds to the EC1 domain of FcαRI in a 2:1 stoichiometry. **(D)** A model of dIgA1 bound to four FcαRI molecules. The FcαRI:IgA1-Fc complex (PDB 1OW0) was superimposed onto the solution structure of dIgA1 published by Bonner et al. (24) (PDB 2QTJ). FcαRI molecules bound to the top or bottom IgA1 antibodies are colored green or yellow, respectively. The C-terminal residue of each receptor is shown in orange or red to illustrate the membrane-proximal region. Kindly provided by Andrew B. Herr, PhD (Cincinnati Children's Hospital).

STRUCTURE AND EXPRESSION OF FCαRI

FcαRI is a member of the Fc receptor immunoglobulin superfamily (32). It is expressed on cells of the myeloid lineage, including neutrophils, eosinophils, monocytes, macrophages, Kupffer cells, and certain DC subsets (18, 33, 34). Additionally, FcαRI expression on human platelets was described (35). Unlike for other Fc receptors, the FcαRI gene is located on chromosome 19 (19q13.4) within the leukocyte receptor cluster (LRC). This region also encodes natural killer cell inhibitory/activatory receptors and leukocyte Ig-like receptors. The FcαRI amino acid sequence has therefore more similarity with these receptors than with other Fc receptors (19, 36). The EC1 and EC2 exons each encode an extracellular Ig-like domain. These domains are folded in an angle of ∼90°. The TM/C exon encodes both the transmembrane domain and a short cytoplasmic tail (**Figure 1C**) (36).

Surface expression of two isoforms of FcαRI has been described on human phagocytes. The a.1 isoform is a 32 kDa single pass transmembrane receptor, which can be extensively glycosylated (six N-linked sites and seven O-linked sites) (37). The molecular weight of the a.1 isoform varies between 55 and 75 kDa on neutrophils and monocytes, while the molecule is heavier (70–100 kDa) on eosinophils due to more extensive glycosylation (21, 37). The a.2 isoform misses 66 nucleotides in the EC2 exon resulting in loss of one O-linked glycosylation site and has a weight of 28 kDa (without glycosylation) (38). The a.2 isoform is solely expressed on alveolar macrophages (38).

The expression of FcαRI was estimated to represent 66,000 and 57,000 molecules on neutrophils and monocytes, respectively (33). Expression level can be modulated by several mediators. On neutrophils, N-formylmethionyl-leucyl-phenylalanine (fMLP), interleukin (IL)-8, tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), and granulocyte-macrophage colony–stimulating factor (GM-CSF) enhanced the expression of FcαRI (39–41). GM-CSF has also an important role in mobilizing neutrophils from the bone marrow (42) and was shown to induce high affinity binding of IgA by human neutrophils (43). Neutrophil FcαRI upregulation was rapid and either induced by *de novo* synthesis or via transport from an intracellular pool to the cell surface (44). On monocytes and monocyte-like cell lines FcαRI expression was enhanced by calcitriol, LPS, TNF-α, GM-CSF, and IL-1β, while downregulation was observed in response to transforming growth factor-beta (TGF-β) or interferon-gamma (IFN-γ) (45, 46). Both monomeric and, to a greater extent, polymeric IgA were able to downregulate FcαRI, possibly due to receptor aggregation, resulting in internalization (47–49).

IgA AND FCαRI

Binding of IgA to FcαRI

FcαRI is a low affinity Fc receptor for monomeric IgA and dIgA ($K_a = 10^6\ M^{-1}$), while IgA immune complexes bind with high avidity and cross-link FcαRI (50). Monomeric IgA binds to the EC1 domain of FcαRI via its Cα2 and Cα3 domains in a 2:1 stoichiometry (i.e., one IgA molecule binds two FcαRI molecules) (**Figure 1C**) (51, 52). Presence of residues Pro440-Phe443 and Leu257-Leu258 in these domains is essential for IgA binding to FcαRI (53).

Dimeric IgA contains four FcαRI binding sites and can therefore theoretically bind four FcαRI, although this is presumably not possible due to steric hindrance (**Figure 1D**) (24). It remains to be elucidated how dIgA exactly interacts with the FcαRI. Binding of SIgA to FcαRI is hampered because of steric hindrance by SC. In order for SIgA to activate cells, co-stimulation of FcαRI, and the lectin Mac-1 (CD11b/CD18) was necessary (54).

Little is known about the difference between IgA1 and IgA2 binding to FcαRI (if any) or the influence of glycosylation on binding capacity. It was however shown that a specific mutation (Asn58 to Glu58) resulted in an altered glycosylation pattern of FcαRI, which increased the binding capacity of IgA nearly 2-fold (55). Removal of sialic acids led to a nearly 4-fold increase of IgA binding. This demonstrates the importance of glycosylation at position 58 of FcαRI in binding affinity for IgA (55). N-glycans located at the external surface of the IgA heavy chain were important for interaction with FcαRI as well (56). Furthermore, it was demonstrated that alterations in IgA1 glycosylation and impaired sialylation of FcαRI were linked to increased binding of IgA1 to FcαRI on neutrophils of patients with IgA nephropathy, which may influence pro-inflammatory functions (47). In transfectants, eosinophils, and monocytes FcαRI binding capacity for IgA immune complexes was enhanced by incubation with several cytokines like GM-CSF, IL-4, and IL-5, without affecting the expression level of the FcαRI on the cell surface (43, 57).

Competitive binding for FcαRI has been described for pentraxins, including the acute phase C reactive protein and serum amyloid P component, resulting in cell activation (58). These proteins are characterized by a pentameric ring-like structure containing five subunits, which recognize a similar site on FcαRI as IgA. However, mutations in FcαRI outside the IgA binding site did not affect IgA binding, but enhanced pentraxin binding 2-fold, suggesting that pentraxins bind to a broader region on FcαRI than IgA (58).

Importantly, *Staphylococcus aureus* and group A and B streptococci developed evasion strategies for IgA-mediated elimination by FcαRI-expressing immune cells by producing

several decoy proteins that obstruct binding of IgA to FcαRI (22, 23). Anti-IgA proteins from group B streptococci include Sir22, Arp4 and an unrelated β protein, whereas *Staphylococcus aureus* produces staphylococcal superantigen-like protein seven. These proteins bind to specific Fc residues (amino acids Pro440-Phe443; i.e. PLAF) in the Cα2 and Cα3 domains of IgA (22, 23, 59). The fact that pathogens evolved and developed mechanisms to evade IgA-mediated elimination by FcαRI-expressing immune cells (60), challenges the dogma of IgA as a non-inflammatory and possibly redundant antibody.

Cell Activation After FcαRI Cross-Linking

Cross-linking of FcαRI by IgA immune complexes (or IgA-opsonized pathogens) induces a variety of processes, including phagocytosis, antibody-dependent cellular cytotoxicity, superoxide generation, release of inflammatory mediators, and cytokines as well as antigen presentation (61–65). Due to hampered binding of SIgA to FcαRI, SIgA does not induce efficient uptake of pathogens by neutrophils or Kupffer cells. However, respiratory burst in neutrophils can be triggered by SIgA, although less efficiently compared to serum IgA (54). Phagocytosis of IgA-coated bacteria or yeast particles by neutrophils was enhanced after priming with GM-CSF or IL-8 (43, 66). Uptake of IgA-coated particles induces increased reactive oxygen species production, subsequently resulting in the release of neutrophil extracellular traps (NETs), which are web-like structures consisting of DNA and proteins (e.g., elastase and myeloperoxidase) able to catch pathogens (61). FcαRI cross-linking furthermore induces release of the neutrophil chemoattractant leukotriene B4 (LTB4) by neutrophils, thereby generating a positive migration feedback loop (64). LTB4 was also shown to mediate migration of monocyte derived DCs (67). As such, in case of mucosal infection neutrophils can function as potent phagocytes, which eliminate invading IgA-coated pathogens, and attract DCs that present antigens to T cells. It was recently shown that *in vitro* generated human mucosal $CD103^+$ DCs (cultured with retinoic acid) express FcαRI. After stimulation with IgA-coated *Staphylococcus aureus* in the presence of Pam3CSK4 $CD103^+$ DCs produced pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-23, which was dependent on FcαRI (68). Furthermore, IgA-stimulated $CD103^+$ DCs enhanced IL-17 production by allogeneic T cells and IL-22 production by intestinal type three innate lymphoid cells, respectively. This suggests that FcαRI crosslinking by IgA induces a pro-inflammatory T helper 17 response accompanied with IL-22 induced activation of epithelial cells, which might contribute to tissue repair (68).

Signaling via FcαRI

In order to initiate optimal effector functions after FcαRI cross-linking, FcαRI needs association with the FcR γ-chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM) in its intracellular domain (69–72). After cross-linking of FcαRI, the src family kinase Fyn aggregates with the receptor complex and phosphorylates the tyrosines in the ITAM in order to act as docking sites for signaling molecules (69, 73, 74). Recruited Syk associates with the phosphorylated ITAM and plays an essential role in the signaling cascade. It directly activates phosphoinositide 3-kinase and phospholipase Cγ, which are involved in two separate signaling routes. These pathways are interconnected and can therefore modulate each other (69, 72, 75). Eventually the signaling pathways will activate the cell, resulting in pro-inflammatory functions (18, 66). Alternatively, it was demonstrated that inhibitory signals can also be transduced via the FcαRI-FcR γ-chain complex. Monomeric targeting of FcαRI (not leading to cross-linking) results in an inhibitory signal and is dependent on Lyn instead of Fyn kinase (74). Phosphorylation of Syk, LAT, and ERK is hampered by "inhibisomes" that contain signaling molecules and both inhibitory and activating receptors (20, 76). Inhibisomes impair activating Fc receptor functioning via a process referred to as inhibitory ITAM (ITAMi) receptor signaling that initiates anti-inflammatory responses to dampen pro-inflammatory responses induced by other Fc receptors (77). Thus, Lyn and Fyn are essential molecules controlling ITAM inhibition and activation, respectively (74). Signaling via FcαRI was recently described in more detail in Aleyd et al. (18).

IgA AND FCαRI MEDIATED FUNCTIONS AT DIFFERENT LOCATIONS

In the Lumen of Mucosal Sites

The functions of SIgA in the intestinal lumen have been well-characterized (**Figure 2A**). SIgA and commensal microbes have important roles in maintaining balance between tolerance toward non-harmful commensals and food compounds vs. immunity against pathogens (78, 79). When SIgA is released from the epithelial layer into the lumen, it remains attached to the outer mucus layer (80). This close localization to bacteria enables SIgA to disturb bacterial motility and to surround pathogens that have a hydrophilic shell. SIgA coating blocks entrance of pathogens into the intestinal epithelium (a process called immune exclusion). This coating leads to agglutination, because antibody-mediated crosslinking of surface antigens will lead to formation of bacterial clumps. Peristaltic bowel movements ensure removal of bacterial aggregates. Bacterial products like enzymes and toxins can be neutralized by SIgA and adherence to host cells, including epithelial cells, is prevented (78). Thus, together with the mucus layer, SIgA forms a barrier against pathogens, and commensals by preventing colonization and penetration of the mucosal epithelium, thereby avoiding infection and antigen leakage into the systemic circulation (81).

Recently the interplay between luminal microbiota and SIgA was investigated in more detail (4, 82) (**Figure 2A**). It is still unclear how SIgA and the microbiota exactly relate to each other, but it was described that SIgA shapes and diversifies the gut microflora on the one hand, while the microbiota on the other hand has an important role in regulating IgA levels (82–84). During weaning up to 70% of intestinal commensals are coated with SIgA in mice and the majority of human fecal bacteria in healthy donors are opsonized with IgA, emphasizing the importance of this association in maintaining homeostasis (31, 85). A wide variety of commensals can be opsonized with

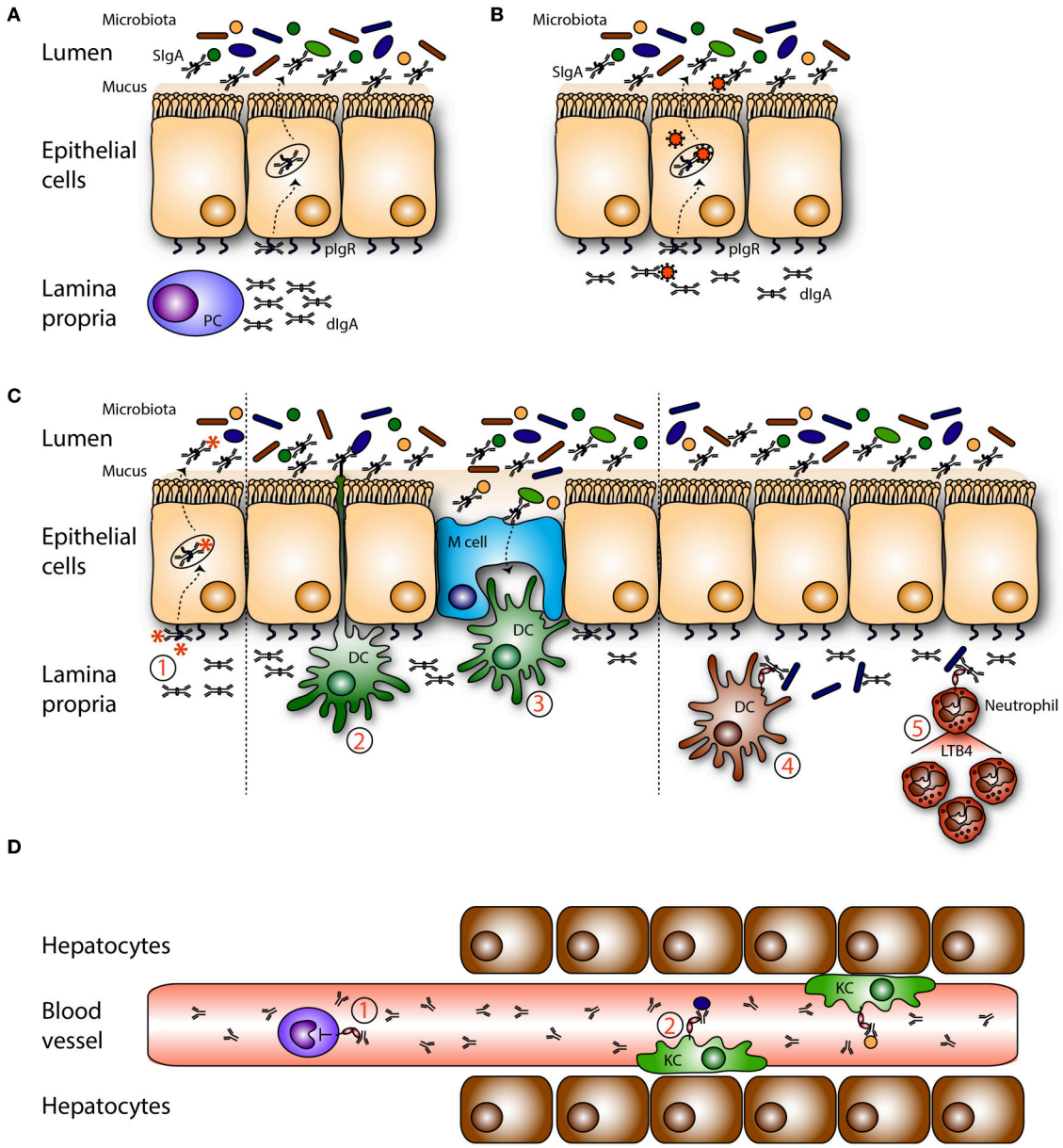

FIGURE 2 | Roles of mucosal IgA in homeostasis. **(A)** Dimeric IgA (dIgA) is produced by local plasma cells (PC) in the lamina propria. Dimeric IgA is transported to the intestinal lumen by binding to the polymeric immunoglobulin receptor (pIgR) present on epithelial cells. In the lumen it is released as secretory IgA (SIgA) where it can coat (commensal) bacteria. **(B)** On route dIgA can bind, neutralize and eliminate viruses. **(C)** (1) Infiltrated antigens and pathogens are opsonized by dIgA and transported back into the lumen. Sub epithelial dendritic cells (DCs) can (2) sample antigens or (3) take up SIgA-coated pathogens that enter via microfold cells (M cells). Pathogens in the lamina propria are coated with dIgA after which this immune complex is taken up by FcαRI- expressing (4) DCs, and (5) neutrophils. In response neutrophils secrete leukotriene B4 (LTB4), hereby attracting more neutrophils, which will clear the infection. **(D)** Serum IgA is (1) capable of inhibiting (unwanted) pro inflammatory responses in the circulation via ITAMi signaling in monocytes. (2) IgA-opsonized bacteria which have leaked into the circulation are taken up by FcαRI-expressing Kupffer cells (KC) in the liver.

polyreactive IgA, although it remains unclear how these IgA antibodies are generated, what the relevant targets are and how binding affects the fitness and physiology of commensals (86). In contrast to commensals, pathogens elicit highly specific T-cell dependent IgA responses (86, 87). Genetic background was shown to influence the repertoire diversity and abundance of innate IgA as germ free (GF) naïve BALB/c mice already had substantial innate IgA levels, independent of microbiota colonization, while this was not the case for C57BL/6 mice (83).

Furthermore, highly glycosylated IgA was able to bind to several gram negative bacteria, which modulated their gene expression, leading to enhanced processing of fibers and

production of butyrate as well as a diversified microbiota composition (82). Moreover, it was shown that the disbalanced microbiota of colitogenic mice became more balanced and diversified after oral administration of the monoclonal IgA W27, which is a high-affinity polyreactive antibody (84). Importantly, mice lacking IgA, J-chain or pIgR were unable to efficiently protect themselves against mucosal infections. These mice have an altered gut microbiota as well as increased mucosal permeability, which renders them more susceptible to develop colitis (80, 88–94). Of note, lack of J-chain or pIgR also led to absence of SIgM which may have contributed to altered microbiota and compromised immunity (88, 91, 94). A diversified microbiota can regulate the amount of SIgA present in luminal secretions by enhancing the level of pIgR on epithelial cells (1, 31, 95), illustrating the complex interrelationship between IgA (and IgM) and the microbiota in maintaining homeostatic conditions.

In Epithelial Layers

It was described that SIgA can be translocated back into the lamina propria (retrograde transport). SIgA and SIgA immune complexes can be transported across specialized epithelial cells, referred to as M cells, which are located in the Peyer's patches of the intestine. Dectin-1 that is expressed by M cells may facilitate transepithelial transport (11, 96). Additionally, a role for TfR1 on epithelial cells in retrotransport of SIgA-coated gliadin was reported in studies investigating celiac disease (12). Retrotransported SIgA immune complexes can be taken up by DCs that are present in the subepithelial dome. *In vitro* models support an essential role for DC-SIGN in recognizing SIgA immune complexes (13). The retrograde transport mechanism and recognition of SIgA by DCs is thought to be essential in monitoring the antigenic status of the intestinal lumen.

On route through epithelial cells, dIgA has the ability to intercept and disarm viruses and redirect them into the lumen, after which they are removed with the feces (**Figure 2B**). Neutralization of several viruses by IgA including sendai, influenza, rota, measles, and human immunodeficiency virus (HIV), as well as bacterial LPS was demonstrated *in vitro* (97–104). IgA has enhanced neutralizing capacity compared to other Ig isotypes due to the N-linked glycosylation of position N459D in the heavy chain Cα3 (105). This effect is independent of the antigen binding site as glycosylated non-specific IgA neutralized the virus as well.

IgA deficient mice, J-chain deficient mice (lacking dIgA, SIgA, and SIgM) and pIgR deficient mice (lacking SIgA and SIgM) have defects in clearing viruses, are more susceptible to reinfection and lack protective immunity (106–108), which emphasizes the important role of IgA in anti-viral immune responses. By contrast, in a recent study pIgR knock-out mice showed a reduced acute norovirus infection rate, suggesting that pIgR and natural polymeric immunoglobulins may promote viral infections. It was suggested that the less diversified intestinal microbiota present in pIgR deficient mice was responsible for reduced infection. pIgR deficient mice had enhanced levels of the anti-viral cytokine IFN-γ, which might explain the reduced infection rates (109).

In the Lamina Propria

Food particles and commensal microbiota are abundantly present in the lumen and can continuously reach the lamina propria through diffusion (via epithelial tight junctions) or transcytosis (**Figure 2C**). These non-harmful components need to be tolerated and they need to be cleared from the mucosa to avoid the formation of immune complexes, which may trigger undesired immune responses. In the lamina propria dIgA can eliminate both non-harmful and harmful components from the tissue by transporting immune complexes back into the lumen through association with the pIgR (97, 101). Additionally, FcαRI$^+$ residing immune cells may help to clear IgA-opsonized pathogens. Under homeostatic conditions, only few FcαRI$^+$ cells are found in mucosal areas (own unpublished data). However, as dIgA-opsonized complexes have the ability to cross-link FcαRI, leading to release of LTB4, neutrophils can be rapidly recruited (64) (**Figure 2C**). Furthermore, *in vitro* generated FcαRI$^+$ CD103$^+$ DCs were shown to produce pro-inflammatory cytokines after stimulation with IgA-coated *Staphylococcus aureus* in the presence of Pam3CSK4, suggesting that a protective adaptive immune response is initiated during mucosal infections (68).

Systemic Protection

It was demonstrated that a variety of commensal microbes in the gut influenced serum IgA levels, which protected mice against lethal sepsis when the intestinal barrier was damaged (110). Serum IgA was shown to target the same epitopes and used the same V-gene segments as plasma cells in the gut (111), suggesting that systemic and mucosal plasma cells originated from the same B cell clone. Diminished serum IgA titers were found in GF mice compared to specific pathogen free mice, supporting the essential role for microbiota diversity in IgA production (110, 112). Mice lacking pIgR and SIgA have epithelial barrier disruption, enhanced numbers of IgA-secreting plasma cells and systemic immune activation indicated by increased levels of serum IgG and IgA (113, 114). Higher levels of albumin were found in the feces, suggesting leakage of serum proteins across the epithelium (113). At the same time intestinal proteins and microorganisms can leak into the tissue and enter the bloodstream. After entering the portal circulation they will encounter Kupffer cells in the liver, which are specialized FcαRI$^+$ macrophages that can eliminate IgA-coated bacteria from the bloodstream (**Figure 2D**) (34). Kupffer cells, macrophages, and monocytes produced increased levels of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) after cross-linking of FcαRI in the presence of pathogen recognition receptor ligands (such as Pam3CSK4, LPS, flagellin) (reflecting IgA-opsonized pathogens) (115).

In contrast to the activating properties of complexed serum IgA, monomeric serum IgA was capable of downregulating cell responses and promoted powerful anti-inflammatory effects (**Figure 2D**). This is referred to as ITAMi signaling (see above) (19). Monomeric targeting of the FcαRI with serum IgA may protect against enhanced receptor activation, which is beneficial in inflammatory diseases characterized by the presence of IgG immune complexes or enhanced FcεRI signaling.

IgA-ASSOCIATED DISEASES

In babies circulating IgA levels are physiologically low because IgA cannot be transported across the placenta. Colostrum and breast milk are important sources of SIgA antibodies to provide local protection against infections. As soon as exposure to microbiota takes place, the SIgA immune system is rapidly maturing. Nonetheless, adult serum IgA levels are not reached before puberty as the systemic IgA compartment develops very slowly. The daily production rate of IgA exceeds that of all other combined antibody classes (66 mg/kg/day), suggesting that the role of IgA in immune defense must be considerable for the body to spend high energy levels (30). Abnormal IgA levels in serum and external secretions (either higher or lower) have been described in numerous pathologies. Very low or absent IgA is referred to as IgA deficiency (116). High levels of (aberrantly glycosylated) IgA are present in multiple diseases including IgA nephropathy, dermatitis herpetiformis, IgA vasculitis, and rheumatoid arthritis.

IgA Deficiency

Selective IgA deficiency is the most common human immunodeficiency and is characterized by a serum IgA concentration of <7 mg/dL. Both serum IgA and mucosal IgA are very low or absent in selective IgA deficient patients (6, 117). Defects in B cell isotype switching, terminal differentiation of IgA^+ plasma cells into secretory cells or long-term survival of IgA-secreting plasma cells can result in IgA deficiency (118, 119). Unbalanced cytokine production (including IL-4, IL-6, IL-7, IL-10, and IL-21) has been reported with a key role for TGF-β, as it induces isotype switching and differentiation of antigen-stimulated B cells into IgA-secreting plasma cells (120, 121). The initial defect may lie in the stem cell compartment as IgA deficiency can be transferred by bone marrow transplantation (122).

A significant heterogeneity exists among IgA deficient patients. Many patients are asymptomatic and only few show minor clinical symptoms. This may in part be due to better hygiene, vaccination, and antibiotic use in modern Western society. Furthermore, it was shown that increased production of IgM and/or IgG can partially compensate for the lack of IgA (**Figure 3A**) (123, 124). IgA deficient patients can develop severe pathology when IgG and/or IgM does not compensate for IgA loss (117, 125). Conflicting reports on the influence of IgA deficiency on fecal microbiota in patients have been reported. One study showed a significantly less diverse fecal microbiota of IgA deficient patients compared to healthy controls, and specific taxa were lost in IgA deficient patients (124). Another study reported a mild loss in microbial diversity in IgA deficient patients (117). SIgM could partially rescue the microbiota composition in IgA deficient patients (117). However, SIgM targeted a broader range of microbes and showed less specificity for microbes compared to SIgA (124). Patients can have increased C reactive protein levels, which is indicative for enhanced inflammation, along with an increased risk of death 10–15 years after initial diagnosis (126, 127). In a recent study no risk association was found between IgA deficiency and hospital infections (127). IgA deficiency was also not more common among hospitalized individuals compared to healthy blood donors (123). However, patients have moderate enhanced susceptibility to gastrointestinal, urinary, and recurrent respiratory infections, allergies, celiac disease, and autoimmune diseases (6, 128, 129). Forty-five percent of IgA deficient patients have the 8.1 haplotype (HLA-A1, B8, DR3, DQ2 haplotype) compared to 16% in the general population (123, 130). The 8.1 haplotype is also commonly found in patients with autoimmune diseases (including rheumatoid arthritis and systemic lupus erythematosus), which are strongly associated with IgA deficiency (123). These findings suggest that patients share predisposing genes, which may explain the increased prevalence of selective IgA deficiency in certain autoimmune disorders.

Eighty-four percent of IgA deficient patients were positive for a wide variety of allergens in skin prick tests (131). The link between IgA deficiency and allergies may be explained by higher levels of circulating antigens due to increased permeability at mucosal surfaces. Alternatively FcαRI may not be able to induce ITAMi signaling in the absence of monomeric serum IgA, possibly resulting in overactivation of immune cells that can lead to development of allergies and autoimmune diseases (132).

Anti-IgA antibodies, usually of the IgG subclass, are commonly found in IgA deficient patients (40%), although their etiology remains unknown. Transfusing IgA-containing blood products to treat IgA deficiency is therefore complicated, as IgA-anti-IgA immune complexes can induce severe reactions, especially when anti-IgA antibodies of the IgE subclass are present (133, 134).

Allergic Diseases

Allergic diseases are inflammatory conditions of which the majority are characterized by high specific IgE levels and activated mast cells (135). Alterations in microbiota diversity reduced immunological tolerance, potentially resulting in food allergies, allergic rhinitis, and asthma (136). It was suggested that the current hygienic life style in the western world altered normal microbiota colonization in infants thereby contributing to the enhanced prevalence of allergies ("hygiene hypothesis") (137). A less diverse composition of the microbiota was associated with lower IgA levels, suggesting that IgA can be involved in the development of allergic diseases as well. Studies investigating the link between IgA levels and allergy and asthma have however been conflicting. In children, serum IgA levels correlated negatively with asthma severity (138). Moreover, it was shown that lower levels of intestinal bacteria were coated with IgA in infants with asthma (139). In adults, both house dust mite sensitization and airway hyper-responsiveness correlated negatively with serum IgA (140). Furthermore, asthma symptoms like shortness of breath and sputum production inversely correlated with, respectively mucosal SIgA and serum IgA levels (141). These results suggest a potential link between low IgA levels and the risk and severity of allergic asthma, which supports a protective role for IgA in allergy. However, data has also been reported showing enhanced specific IgA levels in patients with allergic rhinitis and atopic asthma

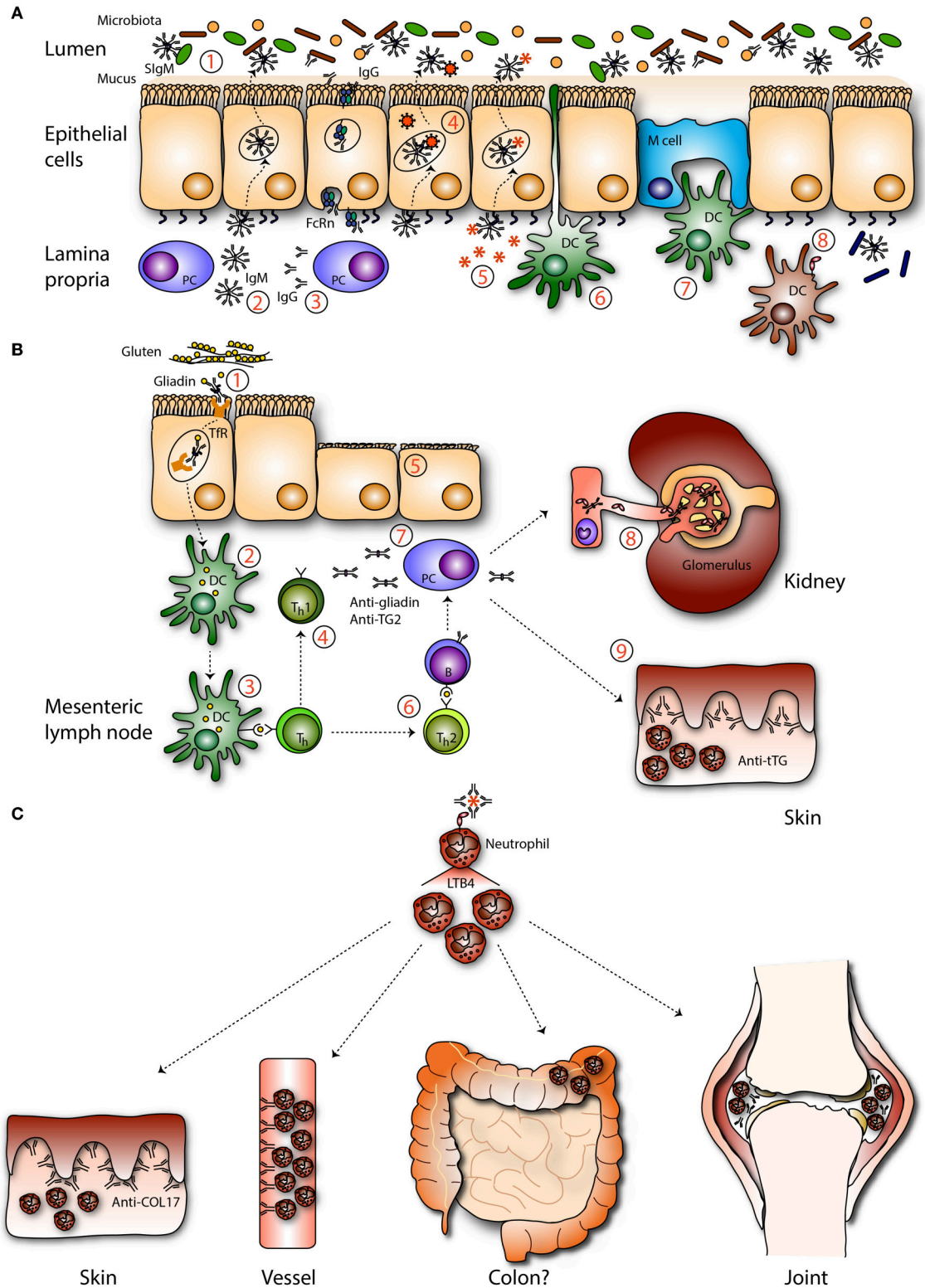

FIGURE 3 | Mucosal IgA in pathogenesis. **(A)** IgA deficiency. In absence of IgA the intestinal microbiota is (1) less diverse and unbalanced. (2) IgM, and (3) IgG can be transported across the epithelium by associating with the pIgR or the neonatal Fc receptor (FcRn), respectively, to compensate IgA loss. Theoretically, IgM may be able to (4) neutralize viruses and (5) exclude infiltrated antigens. Sub epithelial DCs are not described to (6) sample IgM coated bacteria. (7)

(Continued)

FIGURE 3 | IgM-coated bacteria are not described to enter M cells. (8) Invaded IgM-coated pathogens are not recognized by FcαRI-expressing immune cells, resulting in less efficient pathogen elimination. **(B)** Celiac disease and gluten-associated diseases. (1) IgA-gliadin complexes bind to the transferrin receptor (TfR) and are retrotransported across the epithelium. (2) In the lamina propria deamidated gliadin is taken up by DCs and after processing (3) presented to T helper (Th) cells. (4) Th1 cell activation leads to the release of pro-inflammatory mediators (5) causing tissue damage. (6) Activated Th2 cells stimulate autoantibody production directed against gliadin and tissue transglutaminase by B cells. (7) Plasma cells produce autoantibodies which (8) can be detected in the circulation and form deposits with soluble FcαRI in the kidney resulting in damage (IgA nephropathy). (9) IgA anti-tissue transglutaminase forms complexes in the dermis, resulting in neutrophil activation and concomitant tissue damage (dermatitis herpetiformis). **(C)** IgA-FcαRI induced pathology. IgA immune complexes activate neutrophils by cross-linking FcαRI, resulting in release of LTB4 and enhanced neutrophil influx, which induces tissue damage as seen in the skin (LABD), vessels (IgA vasculitis), joints (RA), and potentially in colon (IBD).

(142, 143). Moreover, allergen specific IgA in combination with eosinophilia were common characteristics of asthma and allergic rhinitis (142), possibly because IgA can induce eosinophil survival, thereby contributing to disease severity (144). These results demonstrate a dual role of IgA in allergies, which is currently ill-understood.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune disorder characterized by infiltration of inflammatory immune cells in the joints, resulting in swelling and pain. Presence of autoantibodies including rheumatoid factor (RF) and anti-citrullinated protein antibodies are commonly present, which can be of the IgM, IgG or IgA isotype. High IgA anti-citrullinated protein antibodies and IgA RF titers correlate with worse disease prognosis and severity, and have been used as predictive value for disease progression (145–147). This suggests that IgA contributes to disease pathology. It was demonstrated that blocking the interaction between IgA RF and macrophage FcαRI resulted in reduced levels of TNF-α (148). Additionally, neutrophils stimulated with plasma of RA patients, containing IgA RF, induced NET release, which was inhibited by blocking FcαRI (149). Thus, IgA-immune complexes in patients can induce pro-inflammatory functions of neutrophils and macrophages, which are prominently present in inflamed joints (150), and as such contribute to inflammation in RA.

IgA Nephropathy

IgA nephropathy (IgAN) is the most common form of glomerulonephritis and progresses to end-stage kidney failure in 50% of patients. IgA levels are increased in both serum and urine of IgAN patients. A link with RA was suggested, as patients can also have enhanced IgA RF in their serum. This condition is referred to as rheumatoid nephropathy (151). It was hypothesized that galactose-deficient O-glycans in IgA1, produced by plasma cells in the gut, can trigger the production of anti-glycan IgG/IgA antibodies, after which formed immune complexes deposit in the glomeruli where renal injury is initiated (152, 153). The enzymes responsible for production of O-linked glycans are glycosyltransferases, which expression are amongst others regulated by bacterial products, suggesting that the microbiome regulates the specific glycosylation pattern of IgA1 during the initial phase of IgAN (154, 155). In mice, galactose-deficient IgA1 was not cleared from the murine circulation and deposited in the kidney (15). FcαRI$^+$ Kupffer cells were unable to clear circulating IgA of IgAN patients *in vivo* (156). In transgenic mice expressing the human FcαRI on monocytes and macrophages, soluble FcαRI-IgA complexes that were deposited in the mesangium, induced glomerular and interstitial macrophage infiltration, hematuria, mesangial matrix expansion, and mild proteinuria (157, 158). Moreover, in FcαRI transgenic and human IgA knock-in mice soluble FcαRI-IgA complexes induced kidney inflammation by interacting with TfR1 on mesangial cells, which induced the release of pro-inflammatory mediators. Additionally, the expression of transglutaminase 2 was induced, which subsequently enhanced the expression of TfR1, thereby inducing a pathogenic amplification loop (157). Of note, shedding of FcαRI from macrophages of transgenic mice may have aggravated disease, which will likely not occur in patients as human macrophages express lower FcαRI levels. Nonetheless, IgA-soluble FcαRI complexes have been found in patients with IgAN, and serum IgA immune complexes bound more avidly to TfR1 *in vitro* compared to those from healthy controls (158, 159). It was demonstrated that IgAN and Henoch-Schönlein purpura nephritis patients had reduced levels of soluble FcαRI and transglutaminase 2 in their urine (160), which makes it plausible that soluble FcαRI immune complexes deposit in the human kidney.

IgA Vasculitis

IgA vasculitis, also known as Henoch-Schönlein purpura, is the most common form of vasculitis. The condition is characterized by IgA1 immune deposits and neutrophil infiltrates affecting the small vessels. As a result, red blood cells can leak into the skin leading to typical cutaneous hemorrhages, which leads to diagnosing the disease (161, 162). The pathology of IgA vasculitis remains unclear. However, it is suggested to represent a systemic equivalent of IgAN, as IgA vasculitis can be accompanied with nephropathy, resembling IgAN (163). Unlike IgAN, which is characterized by deposits of galactose-deficient IgA1, it is unknown which type of IgA accumulates in IgA vasculitis (152, 164).

Increased levels of soluble FcαRI-IgA complexes were found in sera of adult and pediatric vasculitis patients with or without co-existing nephritis, which was associated with decreased FcαRI expression on monocytes (165, 166). Furthermore, it was proposed that IgA1 anti-endothelial cell antibodies might play a role. Serum IgA from IgA vasculitis patients was shown to bind *in vitro* to human but not bovine glomerular endothelial cells (167). IgA anti-endothelial cell antibodies induced the production of IL-8 by endothelial cells, thereby contributing to an inflammatory environment and neutrophil recruitment

(168, 169). In addition to enhanced levels of serum TNF-α, which promoted anti-endothelial cell antibody binding to endothelial cells, IL-8 production increased inflammation in IgA vasculitis patients (167, 169, 170). It was hypothesized that neutrophils become activated by IgA-FcαRI mediated cross-linking, resulting in inflammatory processes like reactive oxygen species production and NET formation. Moreover, IgA-activated neutrophils release LTB4, inducing neutrophil migration, thereby enhancing a positive feedback loop, which may result in pathogenesis observed in IgA vasculitis (162).

Inflammatory Bowel Disease

Inflammatory bowel disease is characterized by chronic inflammation in the intestinal tract and is subdivided into ulcerative colitis and Crohn's disease (171). The barrier function in these patients is disrupted allowing bacteria to invade the subepithelial lamina propria (171). Downregulation of pIgR on intestinal epithelial cells and a disturbed microbiota were observed in IBD patients (172). It was proposed that intracellular signaling pathways and protein trafficking was altered due to damage caused by the inflammatory environment resulting in pIgR regulation defects (10). Mice lacking pIgR have enhanced numbers of IgA-secreting plasma cells, possibly as compensation. Increased production, combined with diminished transcytosis of dIgA into the lumen may potentially result in accumulation of dIgA in the lamina propria. Invading bacteria can become opsonized with dIgA, potentially resulting in activation of neutrophils by cross-linking of FcαRI, leading to tissue damage. Neutrophils that had taken up IgA were observed in the mucosa of ulcerative colitis patients (64). Cross-talk between Fc receptors and TLRs was induced by antibody-coated bacteria, resulting in release of pro-inflammatory mediators (e.g., LTB4) by DCs, which may also contribute to inflammation in IBD. LTB4 induces recruitment of neutrophils and monocytes. Additionally, simultaneous triggering of FcαRI and TLR4 on neutrophils resulted in enhanced release of pro-inflammatory TNF-α (173). Furthermore, due to diminished SIgA levels in the lumen, neutralization, and immune exclusion of microbes is decreased, which may worsen disease in patients (10). Patients have increased levels of specific IgA against microbiota in their serum (174). Highly IgA-coated bacteria obtained from IBD patients induced colitis in GF mice (175). The extent to which IgA responses against microbiota differ in homeostatic conditions vs. IBD, remain unclear (86).

Surprisingly, an increased amount of fecal bacteria of IBD patients was opsonized with IgA compared to those obtained from healthy control feces (85, 175, 176). It was suggested that leakage of serum IgA or dIgA due to a disrupted epithelial layer contributed to enhanced fecal bacteria coating (176, 177). The exact role of IgA and FcαRI in inflammatory bowel disease however remains to be elucidated.

Celiac Disease and Dermatitis Herpetiformis

Celiac disease is a multifactorial autoimmune disease characterized by a damaged small intestinal mucosa and nutrient malabsorption following gluten ingestion. Individuals carrying the DQ2 and DQ8 HLA haplotypes have an increased risk to develop celiac disease (178). Gliadin, a glycoprotein of gluten, can complex with specific IgA, after which it can be retrotransported across the epithelium via the TfR (**Figure 3B**). Celiac disease patients have increased expression of TfR on their epithelial cells, allowing increased retrotransport (12). Intracellular degradation of these peptides is disturbed in patients and after entrance in the lamina propria gliadin peptides are deamidated by tissue transglutaminase after which they are presented to HLA-DQ2 or HLA-DQ8 expressing $CD4^+$ T cells. This results in the release of pro-inflammatory mediators, concomitant tissue damage and production of autoantibodies (179). IgA anti-tissue transglutaminase antibodies play a key role in disease pathogenesis (180). Presence of these antibodies serve to diagnose the disease and are furthermore linked to other IgA-related diseases like dermatitis herpetiformis and IgA nephropathy (**Figure 3B**). In dermatitis herpetiformis aberrant IgA antibodies are directed against structural proteins maintaining cell-cell adhesion in the epidermis leading to skin tissue damage (181). It is hypothesized that celiac disease patients can develop dermatitis herpetiformis as high avidity IgA anti-tissue transglutaminase antibodies form immune complexes and deposit in the dermis of these patients, although it is ill understood why some patients develop dermatitis herpetiformis, and others do not. Furthermore, small bowel inflammation in celiac disease is mostly the result of mononuclear cell infiltration, whereas neutrophils accumulate in the skin of dermatitis herpetiformis patients (181). Patient neutrophils have increased ability to bind IgA without FcαRI expression alteration, supporting that FcαRI is primed (e.g., by cytokines or differential glycosylation) (182).

Gluten exposure can also lead to glomerular IgA deposition and IgAN (183). Transgenic human IgA and FcαRI knock-in mice developed severe pathology when gluten were present in their diet, including intestinal injury, increased IgA1–soluble FcαRI complexes, mesangial IgA1 deposition, and elevated serum IgA1 anti-gliadin antibodies. IgAN patients also have increased IgA1 anti-gliadin antibodies in their serum, suggesting that entrance of gluten can potentially result in loss of oral tolerance thereby contributing to IgAN (184). Consumption of a gluten-free diet can resolve manifestations of celiac disease, dermatitis herpetiformis, and IgAN.

Linear IgA Bullous Disease

Linear IgA Bullous Disease (LABD) patients suffer from extensive skin damage and blister formation caused by the presence of IgA autoantibodies against collagen XVII, and concomitant neutrophil accumulation (**Figure 3C**). Collagen XVII plays a critical role in maintaining adhesion between dermis and epidermis in the skin (185). Neutrophil activation is likely the direct result of FcαRI triggering by IgA autoantibodies (186). Tissue damage was induced in the presence of neutrophils and serum of LABD patients (containing IgA anti-collagen XVII autoantibodies) in an *ex vivo* skin model, as presence of activated neutrophils led to separation of the dermis from the epidermis (reflecting blister formation) (186). Eosinophil influx has been observed in the skin of LABD patients as well, suggesting that these cells might also contribute to disease pathology through FcαRI mediated respiratory burst activity (185).

IGA AND FCαRI AS THERAPY

IgA plays an important role in dampening mucosal infections, but can also have detrimental effects in inflammatory or autoimmune diseases. Lack of IgA may increase susceptibility to infection, whereas overabundant IgA complexes or autoantibodies can be harmful as enhanced activation of the FcαRI may lead to various pathologies. Several therapeutic strategies have been proposed that influence the IgA-FcαRI axis (**Figure 4**). For instance, in order to benefit from increased IgA levels during infections it was proposed to increase pathogen-specific IgA levels through passive or active vaccination thereby resulting in efficient clearance of pathogens. Furthermore, IgA might be used as a therapeutic antibody to mediate efficient tumor cell killing. Additionally, two different strategies of targeting FcαRI have been proposed. First, the induction of ITAMi signaling via FcαRI may dampen inflammation that is caused by other pro-inflammatory receptors (e.g., FcεRI). Second, blocking FcαRI may reduce activation of immune cells in IgA-mediated inflammation.

IgA in Vaccination Strategies

Administration of specific IgA (passive immunization) or enhancing IgA production through active immunization might be effective strategies to combat viral and bacterial infections (104). Intranasal passive administration of either specific monomeric or polymeric IgA against a mycobacterium tuberculosis antigen led to short, but effective protection against infection in mice (187). The short protection duration was probably due to degradation of IgA by bacterial proteases present in the respiratory tract fluid. Furthermore, mice expressing human FcαRI on blood neutrophils and monocytes had a lower mycobacterium tuberculosis infection rate compared to control mice after inoculation of human IgA mAb, supporting an additive beneficial role for FcαRI in clearing the infection (188). Wild type (WT) mice which orally received *Salmonella typhimurium* that had been complexed with human plasma-derived IgA and IgM had reduced intestinal infection rates compared to mice exposed to *Salmonella typhimurium* only. Enhanced bacterial clearance was observed when SC was coupled to plasma-derived IgA and IgM (189). Polymeric IgA against influenza virus reached the nasal mucosa after intravenous administration and protected WT mice against infection, and was 10 times more effective than IgG in reducing viral shedding (190, 191). Passive immunization with recombinant dIgA showed better prevention against intra-rectal simian HIV transmission in rhesus macaques. Dimeric IgA could either directly neutralize simian HIV or the virus was trapped by large immune complexes preventing entrance in the epithelial barrier (103). It was demonstrated that IgA isolated from serum of HIV survivors and vaccinated HIV patients neutralized HIV through occupying the CD4 binding site of the virus (192, 193). By contrast, in another study, HIV specific IgA levels in plasma interfered with IgG effector functions *in vitro*, thereby increasing HIV infection risk (194). Recently, it was reported that oral and nasal administrated HIV antigens bound to SIgA were retro transported through M cells and reached sub-epithelial DCs, thereby inducing both mucosal and systemic humoral and cellular immune responses in CX3CR1/GFP transgenic mice (195). Vaccination with SIgA-HIV antigen protected these mice from infection after challenge with a recombinant vaccinia virus expressing the HIV antigen (195, 196). This demonstrates the potential of SIgA to serve as a vaccine carrier for HIV via mucosal administration.

Vaccination with live attenuated polio induced intestinal IgA production and induced long-lived memory immune responses in elderly who still had detectable serum and salivary IgA levels (197–199), which suggested that priming with live virus is needed to obtain IgA responses after booster vaccination with inactivated polio virus. Furthermore, WT mice that were immunized and re-challenged with reovirus had enhanced serum and intestinal IgA levels and were protected from infection (108, 200). $IgA^{-/-}$ mice were unable to clear reovirus infection (108). Mice immunized with hemagglutinin (surface protein on influenza virus) produced anti-influenza specific SIgA, which protected mice from infection after intranasal administration (201). Mice deficient of IgA, J-chain or pIgR showed compromised immune protection (106–108), supporting that IgA enhances protective immunity against viral and bacterial infections.

FcαRI as Therapeutic Target in Cancer Antibody Therapy

Antibodies targeting specific tumor-associated antigens like epidermal growth factor receptor (EGFR) and human epidermal growth factor two (HER2) are increasingly used to treat solid tumors (respectively colorectal cancer and breast cancer) (202). These therapeutic antibodies are often of the IgG isotype and have long half-life, can activate complement, and recruit natural killer cells as well as macrophages as cytotoxic effector cells (202, 203). Alternatively, IgA or FcαRI bispecific antibodies (BsAb) may represent promising novel drugs to treat cancer by enhancing activation of FcαRI-expressing immune cells (204). For instance, BsAb targeting both tumor antigens and FcαRI efficiently recruited neutrophils *in vitro*, which was not observed after targeting Fcγ receptors (205–207). Moreover, (immature) neutrophils were able to kill tumor cells more efficiently due to FcαRI-induced antibody dependent cellular cytotoxicity (62, 203, 208). Similarly, IgA anti-tumor mAbs mediated tumor killing more efficient compared to IgG mAbs. *In vitro* studies demonstrated the superior ability of FcαRI to induce neutrophil-mediated tumor cell killing for multiple tumor antigens, including EGFR, HER2, EpCAM, HLA-II, CD20, CD30, and carcinoembryonic antigen (203, 205, 209).

Unfortunately, *in vivo* tumor targeting using IgA anti-tumor mAbs has been difficult as mice do not express a homolog of the human FcαRI, and human IgA has a short half-life in mice (158). The generation and utilization of FcαRI transgenic mice (71, 158) can contribute to study IgA as therapeutic antibody *in vivo*. Treatment with IgA anti-HER2 or IgA anti-EGFR anti-tumor mAbs resulted in significantly enhanced anti-tumor cytotoxicity in FcαRI transgenic mice compared to WT littermates, which was mediated by FcαRI-expressing macrophages (210, 211). Furthermore, IgA anti-CD20

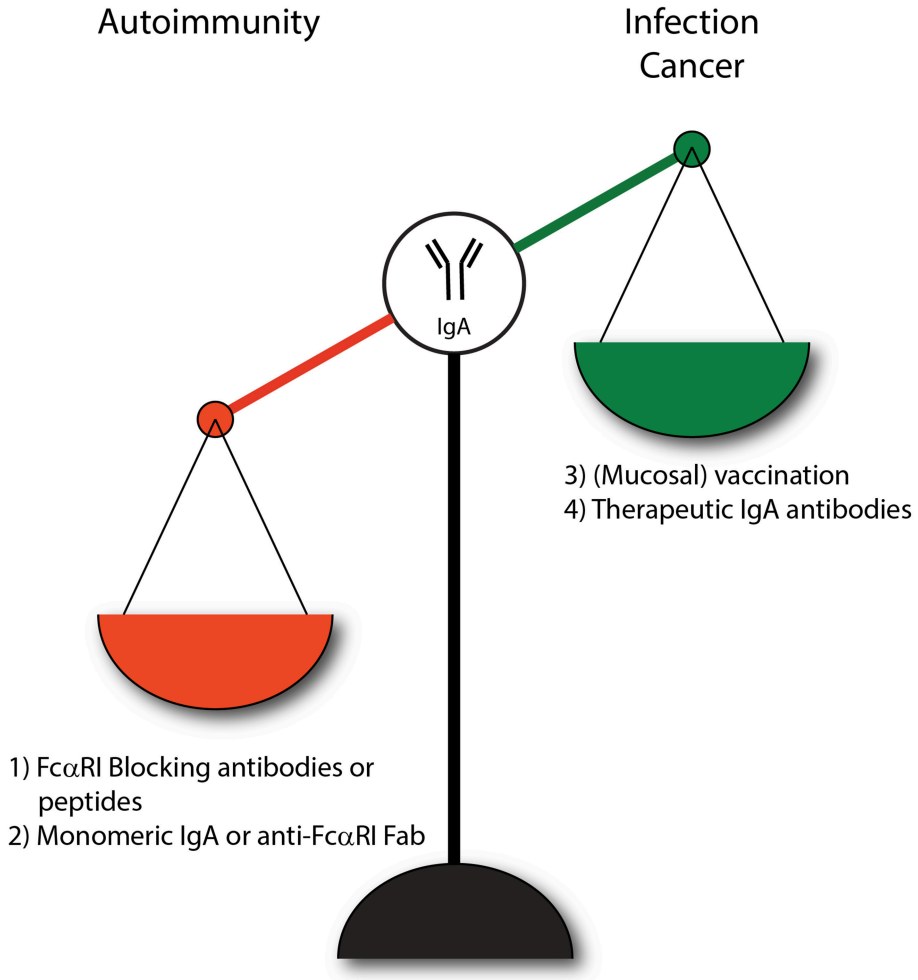

FIGURE 4 | IgA and FcαRI as therapeutic targets. (1) Enhanced IgA-FcαRI activation in IgA-associated autoimmune diseases is unwanted. Blocking IgA-FcαRI interactions by monoclonal antibodies or peptides may reduce tissue damage in these diseases. (2) Treatment with monomeric IgA or anti-FcαRI Fabs may induce ITAMi signaling thereby inhibiting IgG-induced phagocytosis and IgE-mediated allergic diseases. (3) To combat infections, administration of IgA (passive vaccination) or induction of IgA levels via active vaccination may result in enhanced protective immunity. (4) IgA monoclonal antibody therapy may result in efficient killing of tumor cells by activating FcαRI-expressing immune cells.

mAbs inhibited B cell lymphoma cell proliferation *in vitro* and recruited FcαRI-expressing immune cells *in vivo* in FcαRI transgenic mice (212, 213).

Thus, IgA antibodies have been shown to recruit neutrophils and macrophages as effector cells, but the short half-life of IgA in serum is a major drawback for use of IgA as therapeutic antibody. Compared to IgG, the glycosylation sites of therapeutic IgA mAbs that are produced in non-human systems (like rodent cells) have higher immunogenic potential (high glycosylation) and therapeutic IgA will be likely cleared efficiently from the human (or murine) circulation. Increasing the sialylation of glycans on IgA mAbs enhanced their serum half-life due to decreased clearance by the ASGPR in the liver (210). Inducing sialylation of IgA mAbs was done by using fully human systems thereby augmenting their *in vivo* therapeutic potential (211, 214–216). Increased sialylation of the N-glycans in IgA anti-HER2 mAbs resulted in significantly reduced tumor growth in FcαRI-transgenic SCID mice bearing BT-474 tumors (214). Addition of an albumin-binding domain to IgA1 (IgA1-albumin) enhanced interaction of the antibody with the FcRn, which extended the half-life of IgA1 *in vivo*. Although *ex vivo* studies showed that IgA1-albumin induced lower maximal tumor cell lysis, enhanced tumor cell killing was observed *in vivo* (211). Another modified IgA molecule against EGFR lacking glycosylation and free cysteine's, and with a stabilized heavy and light chain linkage, showed increased efficacy by interacting with FcαRI-expressing myeloid cells *in vivo*. Some therapeutic activity was observed in non-FcαRI transgenic mice demonstrating contribution of Fab-mediated effector functions as well (216). Although IgA antibodies are not used in clinical studies yet, engineering IgA with increased half-life represents a promising strategy for targeting tumors in patients.

Induction of ITAMi Signaling

Enhancing ITAMi signaling by monomeric targeting of the FcαRI may be a promising strategy to inhibit IgG-induced phagocytosis and IgE-mediated allergic diseases (20). Monovalent targeting of FcαRI with the anti-FcαRI mAb A77 inhibited degranulation of RBL-2H3 transfected cells and reduced airway inflammation in FcαRI transgenic mice after crosslinking FcεRI with IgE immune complexes in an allergic asthma model (20, 217). Furthermore, naturally occurring serum IgA dampened immune responses by inducing ITAMi signaling via FcαRI (19). FcαRI transgenic mice (expression on monocytes/macrophages), which developed RA after injection with IgG anti-collagen had reduced manifestations or even complete resolution of arthritis after treatment with monomeric IgA. IgG-induced ITAM signaling was blocked efficiently, implicating a potential role of monomeric IgA in treatment of autoimmune diseases with IgG autoantibodies (218). Moreover, prior targeting of FcαRI by Fab A77 suppressed inflammation in transgenic mice with FcαRI-expressing monocytes and macrophages that suffered from IgG immune complex glomerulonephritis and obstructive nephropathy (219). Renal inflammation induced by pristane was characterized by enhanced serum IgG levels, pro-inflammatory cytokines and immune infiltration in FcαRI transgenic mice with receptor expression on monocytes and macrophages. Blocking FcαRI with Fab MIP8a inhibited cytokine production, leukocyte recruitment and inflammation (77). Furthermore, renal inflammation induced by CpG (TLR9 agonist) in these FcαRI transgenic mice was downregulated by monomeric occupancy of FcαRI (220). Thus, monomeric targeting of FcαRI is suggested to induce anti-inflammatory properties, which could be useful in treatment of inflammatory diseases with involvement of myeloid cells.

FcαRI Blocking

Blocking FcαRI might be effective in IgA-mediated inflammation. *In vitro* it was shown that neutrophils stimulated with IgA immune complexes obtained from RA patients released neutrophil extracellular traps. FcαRI blocking on neutrophils with the anti-FcαRI mAb MIP8a reduced NET formation and might alleviate neutrophil induced tissue damage in RA patients (149). IgA autoantibodies in serum of LABD patients induced neutrophil-mediated tissue damage in an *ex vivo* human skin model. FcαRI blocking with MIP8a prevented IgA induced tissue damage (186). Similarly, peptides targeting the interaction sites of IgA and FcαRI showed effective blocking of IgA binding to FcαRI, and reduced IgA-induced neutrophil migration *in vitro*. These peptides were able to penetrate into human skin, supporting that they might function as novel therapy in skin autoimmune diseases (221). As such, blocking FcαRI might serve as therapeutic strategy for IgA-associated inflammatory diseases.

CONCLUSION

IgA is important in maintaining balance of mucosal immunity. SIgA regulates immune exclusion by neutralizing pathogens and more recently the role of SIgA in diversifying the intestinal microbiota has become clear. The mechanisms involved in IgA-mediated regulation of microbiota diversity and reciprocal regulation of IgA levels by microbes are incompletely understood. Moreover, it remains unclear why certain SIgA coated antigens associate with the epithelial layer while others are eliminated via immune exclusion. In addition to the traditional role of IgA as non-inflammatory regulator of homeostasis, several pro-inflammatory functions have been described, which need to be clarified in more detail.

After binding to FcαRI, IgA plays important roles in pathogen elimination. It can also have detrimental effects on human health when aberrant IgA is present. Perpetual IgA-FcαRI interaction results in enhanced activation of immune cells with concomitant tissue damage as seen in autoimmune diseases like LABD (64). It is yet undefined why neutrophil influx is observed in some IgA-FcαRI mediated diseases, including LABD and dermatitis herpetiformis, but not others. For instance, Crohn's disease is generally less characterized by neutrophil influx in the intestinal mucosa, while in patients with ulcerative colitis massive neutrophil infiltration is present (171). Similarly, and maybe even more confusing, is the fact that celiac disease patients develop IgA anti-tissue transglutaminase antibodies resulting in infiltration of mainly mononuclear cells in the intestinal tract, while in dermatitis herpetiformis (skin manifestation of celiac disease) binding of IgA autoantibodies to epidermal transglutaminase results in neutrophil recruitment (181).

Targeting the IgA-FcαRI axis with blocking monoclonal antibodies or peptides may alleviate inflammation and concomitant tissue damage. Moreover, inhibitory ITAMi signaling induced by monomeric targeting of FcαRI has been suggested to represent a promising strategy in allergies or IgG immune complex-mediated diseases. By contrast, in infectious diseases and cancer enhancing pro-inflammatory effects of IgA-FcαRI interaction might be very beneficial.

Lack of an FcαRI equivalent in mice has hampered our understanding of the functions and therapeutic applications of IgA and FcαRI. Future research will be facilitated by the use of several human FcαRI transgenic and human IgA knock-in models (71, 158, 222). This will help to increase our knowledge on the complex roles of IgA and FcαRI in (patho)physiology as well as the therapeutic possibilities for targeting these multifaceted molecules.

AUTHOR CONTRIBUTIONS

AB wrote the paper and designed the figures. MvE designed and illustrated the figures, supervised, and revised the paper.

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An Engineered Human Fc variant With Exquisite Selectivity for FcγRIIIa$_{V158}$ Reveals That Ligation of FcγRIIIa Mediates Potent Antibody Dependent Cellular Phagocytosis With GM-CSF-Differentiated Macrophages

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IgG antibodies mediate the clearance of target cells via the engagement of Fc gamma receptors (FcγRs) on effector cells by eliciting antibody-dependent cellular cytotoxicity and phagocytosis (ADCC and ADCP, respectively). Because (i) the IgG Fc domain binds to multiple FcγRs with varying affinities; (ii) even low Fc:FcγRs affinity interactions can play a significant role when antibodies are engaged in high avidity immune complexes and (iii) most effector cells express multiple FcγRs, the clearance mechanisms that can be mediated by individual FcγR are not well-understood. Human FcγRIIIa (hFcγRIIIa; CD16a), which exists as two polymorphic variants at position 158, hFcγRIIIa$_{V158}$ and hFcγRIIIa$_{F158}$, is widely considered to only trigger ADCC, especially with natural killer (NK) cells as effectors. To evaluate the role of hFcγRIIIa ligation in myeloid-derived effector cells, and in particular on macrophages and monocytes which express multiple FcγRs, we engineered an aglycosylated engineered human Fc (hFc) variant, Fc3aV, which binds exclusively to hFcγRIIIa$_{V158}$. Antibodies formatted with the Fc3aV variant bind to the hFcγRIIIa$_{V158}$ allotype with a somewhat lower K_D than their wild type IgG1 counterparts, but not to any other hFcγR. The exceptional selectivity for hFcγRIIIa$_{V158}$ was demonstrated by SPR using increased avidity, dimerized GST-fused versions of the ectodomains of hFcγRs and from the absence of binding of large immune complex (IC) to CHO cells expressing each of the hFcγRs, including notably, the FcγRIIIa$_{F158}$ variant or the highly homologous FcγRIIIb. We show that even though monocyte-derived GM-CSF differentiated macrophages express hFcγRIIIa at substantially lower levels than the other two major activating receptors, namely hFcγRI or hFcγRIIa, Fc3aV-formatted Rituximab and Herceptin perform ADCP toward CD20- and Her2-expressing

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cancer cells, respectively, at a level comparable to that of the respective wild-type antibodies. We further show that hFcγRIIIa activation plays a significant role on ADCC by human peripheral monocytes. Our data highlight the utility of Fc3aV and other similarly engineered exquisitely selective, aglycosylated Fc variants toward other hFcγRs as tools for the detailed molecular understanding of hFcγR biology.

Keywords: Fc engineering, ADCP, FcgR, macrophage, monocyte

INTRODUCTION

Antibodies regulate a variety of immune responses by engaging Fcγ receptors (FcγRs) expressed on various leukocytes such as macrophages, granulocytes, dendritic cells (DCs), natural killer (NK) cells, and B cells. Immune complexes (ICs) formed by antibodies binding to multivalent antigens such as viruses and antibody-opsonized cancer or infected cells, induce activating FcγR-mediated immunoreceptor tyrosine-based activation motif (ITAM) signaling in effector cells, thereby inducing antibody-dependent cellular cytotoxicity and phagocytosis (ADCC/P) (1, 2). The activation of immune cells upon binding to ICs is regulated by the immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling of the inhibitory FcγRIIb (3, 4). The ensuing signaling processes and immunological mechanisms triggered by IC binding depend on the expression level of FcγRs on various immune cell subsets and on the binding affinity of the Fc domain to the different FcγRs (2, 5). The composition of the single glycan at N297 of the human IgG1 Fc domain is known to both affect human FcγR (hFcγR) receptor affinity and, in some instances, to trigger additional signaling pathways via binding to the lectin-like type II receptors (6).

Human FcγRIIIa (hFcγRIIIa; CD16a) has been reported to be the most potent receptor for mediating ADCC (7, 8). NK cells which are considered to be the most important contributor to ADCC with peripheral blood mononuclear cells (PBMCs) as effector cells, express only hFcγRIIIa (2). hFcγRIIIa is also expressed in intermediate ($CD14^{hi}/CD16^{+}$) and non-classical ($CD14^{dim}/CD16^{hi}$) blood monocytes which are capable of inducing ADCC primarily via TNFα secretion (9, 10). Among the four human IgG subclasses, IgG1 and IgG3 have the higher affinity for hFcγRIIIa resulting in more potent ADCC *in vitro* (5, 11–13). A single nucleotide polymorphism of hFcγRIIIa at residue 158 which can be either Val and Phe is prevalent in humans. Earlier studies revealed that lymphoma patients expressing the higher affinity $hFc\gamma RIIIa_{V158}$ variant show improved clinical outcomes when treated with anti-CD20 (rituximab) and anti-Her2 (trastuzumab) compared to patients homozygous for the lower affinity, $hFc\gamma RIIIa_{F158}$ (14–17). The finding that higher affinity to hFcγRIIIa may lead to greater therapeutic potency stimulated extensive efforts to engineer hFc domains with improved binding hFcγRIIIa via either site-directed mutagenesis or glycoengineering, the latter accomplished primarily by completely abolishing or by reducing fucosylation (18–21). hFc defucosylated antibodies have up to 50-fold enhanced affinity to hFcγRIIIa and three defucosylated antibodies, anti-CCR4 mogamulizumab, anti-IL-5Ra benralizumab, and anti-CD20 obinutuzumab, have been evaluated for multiple therapeutic indications and approved for clinical use (21, 22).

Earlier reports had suggested that the improved therapeutic efficacy of antibodies having increased hFcγRIIIa affinity is due to more effective priming and activation of NK cells (8, 23, 24). However, in more recent studies it was observed that the depletion of macrophages, which are the predominant mediators of ADCP in tissues (25), abrogated the therapeutic efficacy of anti-CD20, anti-CD30, or anti-CD40 antibodies in mouse models whereas removal of NK cells or neutrophils did not significantly affect therapeutic efficacy (26–29). These results underline the critical role of tissue-resident macrophages and ADCP in anti-tumor antibody immunotherapy. ADCP is mainly attributed to signaling via hFcγRIIa, which is expressed at high levels on macrophages (30–32). Indeed, hFc-engineered antibodies with increased hFcγRIIa binding confer more potent ADCP (29, 31). Conversely, glycoengineered antibodies do not display higher affinity toward hFcγRIIa (33), but have been shown to increase macrophage phagocytic activity (34), potentially through hFcγRIIIa signaling.

hFcγRIIIa is expressed at much lower levels than hFcγRIIa in human *in vitro* monocyte-derived GM-CSF differentiated macrophages (gmMϕ) (30–32, 35). Thus, the importance of hFcγRIIIa engagement in mediating ADCP is an overlooked issue (36–38). Delineating the role of a particular hFcγR by blocking other receptors with antibodies can be problematic for two reasons: First, *in vitro* the cross-linking of the targeted hFcγR by blocking antibodies can alter the distribution of receptors on the membrane and also the blocking antibodies may impede the accessibility of other FcγR impacting the binding of immune complexes to other hFcγRs. Secondly, the co-administration of hFcγR blocking antibodies can complicate the design and interpretation of *in vivo* experiments. To circumvent these limitations our lab has been developing engineered Fc domains that have absolute selectivity for only one Fc binding protein (39, 40). The binding of IgG antibodies to hFcγRs or to the C1q component of the complement system is critically dependent on N-linked glycan at residue N297 of the Fc (41). Loss of the glycan increases the conformational flexibility of the CH2 domain resulting in very significant, albeit not complete loss of hFcγR and C1q binding and in drastically diminished effector functions (39, 42–44). Previously, our lab has developed an Fc variant, Fc5, that binds only to the high affinity hFcγRI and not to any other human FcγR. We further demonstrated that antibodies formatted with the Fc5 domain potentiate effective

tumor cell death by monocyte-derived DCs via the ligation of FcγRI (39). In this work, we report on the engineering of an aglycosylated hFc domain, Fc3aV, that has essentially absolute specificity for the hFcγRIIIa$_{V158}$ allotype. Using antibodies equipped with the Fc3aV variant, we showed that exclusive engagement of hFcγRIIIa results in potent ADCP with gmMϕ and also established the role of this receptor on ADCC with monocytes as effectors.

MATERIALS AND METHODS

Cells And Reagents

Burkitt's lymphoma Raji cells (ATCC® CCL-86™) and SK-BR-3 breast tumor cell lines (ATCC® HTB-30™) were obtained from American Type Culture Collection. Raji cells were cultured in complete RPMI with 10% fetal bovine serum (FBS) and SK-BR-3 cells were cultured in complete DMEM with 10% FBS. The collections of CHO cells expressing FLAG-tagged hFcγRs were described previously (14, 40, 45).

Human PBMCs and neutrophils were purified from anonymous healthy volunteers using Histopaque density gradient centrifugation (Sigma-Aldrich). Neutrophils were activated with 50 ng/mL hIFNγ for 24 h. NK cells were isolated by negative immunodensity isolation using the RosetteSep Human NK Cell Enrichment Cocktail (StemCell Technologies). Human GM-CSF differentiated macrophage cells were differentiated from CD14$^+$ monocytes with 50 ng/mL GM-CSF (BioLegend®) for 7 days. Ectodomains of hFcγRI, hFcγRIIa, hFcγRIIb, and hFcγRIIIa, were produced in Expi293 cells (Invitrogen). All primers were synthesized by Integrated DNA Technologies.

Library Screening for hFcγRIIIa-Selective hFc Variant

As reported previously (40), *E. coli* cells expressing an IgG-library (E-library) were cultured overnight at 37°C with 250 rpm shaking in Terrific Broth media (TB, Difco™) with 2% (w/v) glucose, antibiotics (35 μg/ml of chloramphenicol and 50 μg/ml kanamycin). Following overnight growth, cells were diluted 1:100 in fresh TB medium and were induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 0.2% L-arabinose. Following incubation at 25°C for 20 h, the library cells were spheroplasted. as described previously (40). Spheroplasts were labeled with 10 nM dimeric hFcγRIIIa$_{V158}$-GST-rPE and 100 nM of unconjugated dimeric hFcγRIIb-GST and sorted using a FACSAria™ flow cytometer. FACS data were analyzed with FACSDiva software. For FACS screening, clones corresponding to the top 1% fluorescent events were isolated. Sorted spheroplasts were resorted immediately after initial sorting. The hFc mutant genes in the resorted spheroplasts rescued by PCR were cloned into *Sfi*I digested pPelB-AglycoT(H)-FLAG vector, and transformed into electro-competent *E. coli* Jude-1 harboring pBAD-AglycoT(L)-His. The resulting transformants were subjected to a next round of sorting and resorting.

Expression and Purification of IgG Antibodies and hFcγRs

IgG1 antibodies and hFcγRs proteins were produced by transient transfection of Expi293F cells (Thermo Fisher Scientific) using the pcDNA3.4 vector (Thermo Fisher Scientific). Antibodies were purified by Protein A high capacity agarose resin (Thermo Fisher Scientific) affinity chromatography. 25× PBS was added to filtered supernatants to a 1× concentration, and the mixture was passed twice over the column. The column was washed with 100 ml of 1x PBS to remove nonspecifically bound proteins. Three milliliters of 100 mM glycine-HCl (pH 2.7) was added to elute the bound proteins, and the elution was immediately neutralized with 1 ml of 1 M Tris (pH 8.0). Samples were buffer-exchanged into pH7.4 PBS using Amicon Ultra-4 (Millipore) spin columns with a 10 kDa cutoff. The purity of purified samples was assessed by 4–20% gradient SDS-PAGE gel (NuSep).

ELISA and SPR Analysis

Fifty microliters of 4 μg/ml of Trastuzumab IgG1 or its hFc variants were diluted in pH7.4 phosphate buffered saline (PBS) buffer and used to coat 96 well polystyrene ELISA plate (Corning) overnight at 4°C. After blocking with 1x PBS (pH 7.4) containing 0.5% BSA for 2 h at room temperature, the plate was washed 4 times with PBS containing 0.05% Tween20 (PBST), and incubated with serially diluted dimeric GST-tagged low affinity hFcγRs and monomeric his-tagged high affinity hFcγRI at room temperature for 1 h. After washing 4 times with the PBST buffer, either 1:10,000 diluted α-GST (GE Healthcare) or α-His antibody HRP conjugate were added. After washing three times with PBST, 50 μL TMB substrate was added per well (Thermo Scientific), 50 μL of 1 M H_2SO_4 was added to neutralize, and the absorbance at 450 nm was recorded.

For SPR analysis, Trastuzumab IgG1 or its variants were individually immobilized on CM5 sensor chips by amine coupling, as recommended by the manufacturer (GE Healthcare). Binding experiments were performed in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant). Serially diluted dimeric GST-tagged low affinity hFcγRs or monomeric his-tagged high affinity hFcγRI were injected at a flow rate of 30 μl/min for 60 s with a dissociation time of 5 min. The chip was regenerated after each run by sequential injection of 50 mM glycine, pH 4.0, 50 mM glycine, pH 9.5, and 3 M NaCl for 1 min each. For each run, a bovine serum albumin (BSA)-coupled surface was used as reference channel. Equilibrium dissociation constants (K_D) for monovalent receptor binding were determined by fitting 1:1 Langmuir model to the data using BIAevaluation 3.2 software (GE Healthcare) and the K_Ds were averaged from three independent experiments ($n = 3$ for hFcγRIIa and hFcγRIIIa, $n = 2$ for hFcγRI and hFcγRIIb). The resulting sensorgrams were fit with a 1:1 Langmuir isotherm model or equilibrium binding model for monomeric hFcγRs using Biaevaluation 3.0 software.

Antibody Binding Activities to Cancer Cells

Raji cells or SK-BR-3 cells (10^5 cells) were incubated with various concentration of antibody variants for 30 min at

4°C and washed with 3% BSA in PBS. Raji- or SK-BR3-bound antibody levels were detected by FITC-conjugated, $F(ab')_2$ Fragment, goat Anti-Human IgG Fc (Jackson ImmunoResearch Laboratories).

Immune Complex Binding Activities to hFcγRs

CHO cells expressing FLAG-tagged hFcγRs were described previously (14, 40, 45). ICs were also generated by mixing 10 μg/ml of Trastuzumab or Trastuzumab-Fc3aV and 5 μg/ml of PE-conjugated $F(ab')_2$ goat anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories) for 30 min at 37°C (14, 40, 45). CHO cells expressing hFcγRs were incubated with ICs for 1 h on ice and cell-bound ICs were detected by flow cytometry on a MACSQuant (Miltenyi Biotech). Data were analyzed with Flow Cytometry Analysis Software (FlowJo). The IC-binding activities to NK cells or neutrophils were also assayed and measured using same method, but using a FACS Calibur (BD Biosciences).

In vitro Cell-Based Assays

FCGR3A SNP Genotyping

As previously described (46, 47), *FCGR3A* genes were specifically amplified using the following primers, 5′-TCC AAA AGC CAC ACT CAA AGA CAG CGC-3′ and 5′-GAT GGT GAT GTT CAC AGT CTC T-3′ and the sequence of amplified *FCGR3A* was analyzed. *FCGR3A* SNP was also confirmed by nested PCR using following primer sets; 5′-TCC AAA AGC CAC ACT CAA AGA CAG CGC-3′ and 5′-CTC TGA AGA CAC ATT TTT ACT CCC AAA-3′ for *FCGR3A* F158, and 5′-TCC AAA AGC CAC ACT CAA AGA CAG CGC-3′ and CTC TGA AGA CAC ATT TTT ACT CCC AAC for *FCGR3A* V158.

ADCC Assays

SK-BR-3 or Raji cells were cultured in complete DMEM or RPMI medium as above and collected by centrifugation at 300x g for 5 min. Cells were washed in PBS and labeled with 4 μM Calcein AM (Invitrogen) in PBS at 37°C with 5% CO_2 for 30 min. Calcein-loaded cancer cells were washed twice, resuspended in RPMI medium, and seeded into a 96-well plate at 10,000 cells/well with various concentrations of IgG variants. As previously described (40, 48), PBMCs and PMNs were isolated from healthy anonymous donors using Histopaque (Sigma-Aldrich). $CD14^+$ monocytes were isolated from PBMCs by magnetic bead separation (EasySep by STEMCELL Inc.) (40). Effector cells were added into a 96-well plate at 100,000 cells/well and the plates were incubated at 37°C with 5% CO_2 for 4 h. Released calcein AM was detected at excitation and emission wavelengths of 485 and 535 nm, respectively. The percent of tumor cell lysis was calculated according to the following formula; 100 × (E-S)/(M-S), where E is the fluorescence of experimental well, S is that in the absence of antibody (tumor cells were incubated with medium and complement alone), and M is that of tumor cells with lysis buffer (Triton X-100 at 2% v/v, SDS 1% w/v, 100 mM NaCl, and 1 mM EDTA).

ADCP Assays

Purified monocytes were differentiated into GM-CSF differentiated macrophages (gmMϕ) by culture for 7 days in RPMI medium containing 10% FBS and 50 ng/ml GM-CSF. Harvested gmMϕ by trypsinization were seeded into 96-well plate at 10^5 cells/well and then cultured in RPMI medium containing 10% FBS overnight. SK-BR-3 or Raji cells, were labeled with PKH67 (Sigma-Adrich) according to the manufacturer's instructions and opsonized by serially diluted IgG variants. IgG-opsonized and PKH-67-labeled cancer cells were added into gmMϕ-attached 96-well plate at 10^4 cells/well. After 2 h at 37°C with 5% CO_2, gmMϕ cells were detached from the plate by Trypsin-EDTA treatment for 20 min. gmMϕ were stained with anti-CD45-APC (BD bioscience) for ADCP assay with SK-BR-3 cells on ice for 15 min. For ADCP assay with Raji cells, gmMϕ were stained with anti-CD14-APC and anti-CD11b-APC (Biolegend) on ice for 15 min. Phagocytosis was evaluated by FACSAria™ (BD Bioscience), and reported as the fraction of double positive cells over the total number of tumor cells in the sample.

Blockade assays for hFcγRIIIa were performed as follows; gmMϕ were pre-incubated with 10 μg/ml of anti-CD16 mAb 3G8 $F(ab')_2$ (Ancell) (49–51) was pre-incubated with gmMϕ for 10 min. IgG-opsonized and PKH-67-labeled cancer cells were then incubated with anti-CD16 mAb 3G8 $F(ab')_2$-coated gmMϕ in RPMI1640 medium without serum. After 2 h at 37°C, the fraction of phagocytosed tumor cells was detected as described above. For all assays, an E:T ratio of 10:1 was used.

Fluorescent images of macrophages phagocytozing cancer cells were obtained by confocal microscopy using calcein AM-loaded Raji cells or SK-BR-3 cells opsonized with 20 μg/ml of antibodies and gmMϕ at 37°C for 1 h. Approximately 1×10^5 labeled cancer cells and 1×10^5 macrophages were co-incubated in 1 ml of RPMI medium. Subsequently, the co-incubated cells were labeled as described above. Phagocytosis was visualized by confocal microscopy using Zeiss LSM 710/Elyra S.1.

RESULTS

Directed Evolution for hFcγRIIIa-Specific Binding hFc Variant

Aglycosylated hFc domains specific for hFcγRIIIa were engineered via the screening of combinatorial libraries expressed in *E. coli* using a well-established full-length IgG-display system in bacteria (36, 40). Briefly, the heavy chain and light chains of IgG (having Trastuzumab Fab arms) were separately expressed by fusing pelB and NlpA, respectively resulting in tethering of IgG on the periplasmic side of the inner membrane. In order to isolate hFcγRIIIA-selective aglycosylated hFc variants, the IgG hFc domain was mutagenized by error-prone PCR and a library of $>10^9$ *E. coli* transformants was generated. The bacterial cells (40) were spheroplasted and the library was screened with 10 nM of dimeric $hFc\gamma RIIIA_{V158}$-GST, conjugated with rPE in the presence of 100 nM of unconjugated hFcγRIIB-GST as a

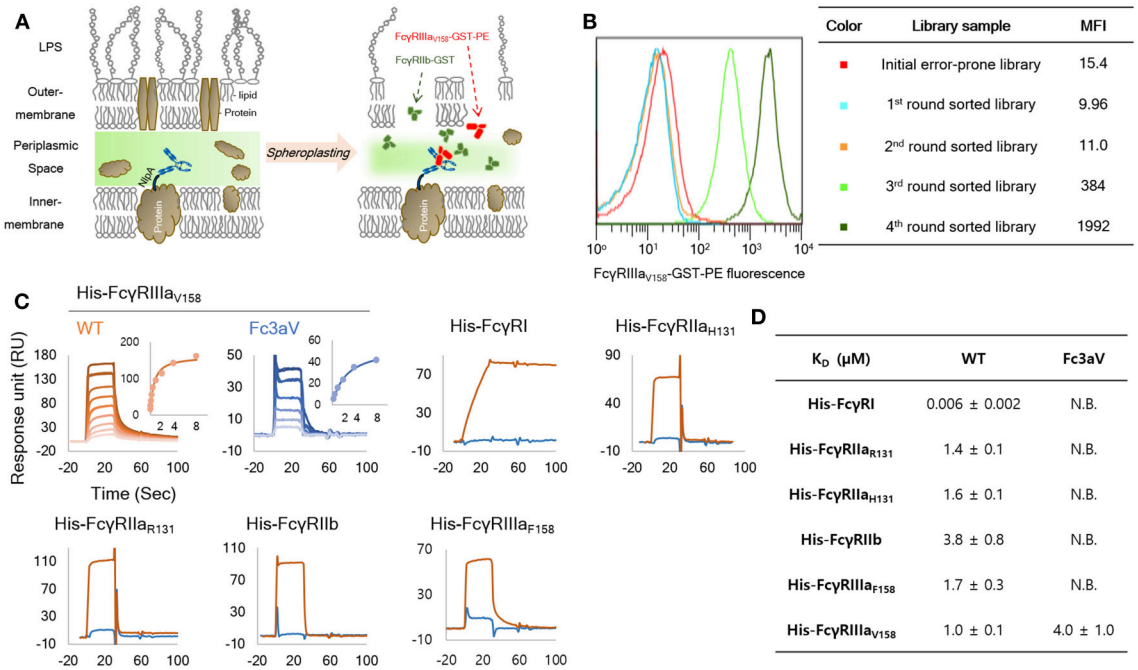

FIGURE 1 | Engineering and biochemical characterization of a of FcγRIIIa$_{V158}$-specific, Fc domain variant: **(A)** Schematic diagram of the high throughput screening system used for the isolation of FcγRIIIa$_{V158}$–specific Fc variants from very large combinatorial libraries. Soluble fluorescent FcγRIIIa$_{V158}$-GST and a large excess of non-fluorescent FcγRIIb-GST as a competitor are used for cell labeling and FACS sorting. **(B)** MFI (median fluorescence intensity) of initial library (red, size: 1 × 10^9), and 1st (light blue), 2nd (orange), 3rd (light green), and 4th (green) round sorted populations. **(C,D)** SPR analysis of wild-type Trastuzumab (orange) and Trastuzumab-Fc3aV (sky blue) with monomeric His-tagged FcγRs. Surface plasmon resonance (SPR) sensorgrams **(C)** and equilibrium dissociation constants for of Fc3aV in μM **(D)**. N.B. means RUmax of sensorgram is lower than 5 RU with 8 μM of each His-hFcγR. All experiments were repeated three times and error corresponds to standard deviation (s.d.).

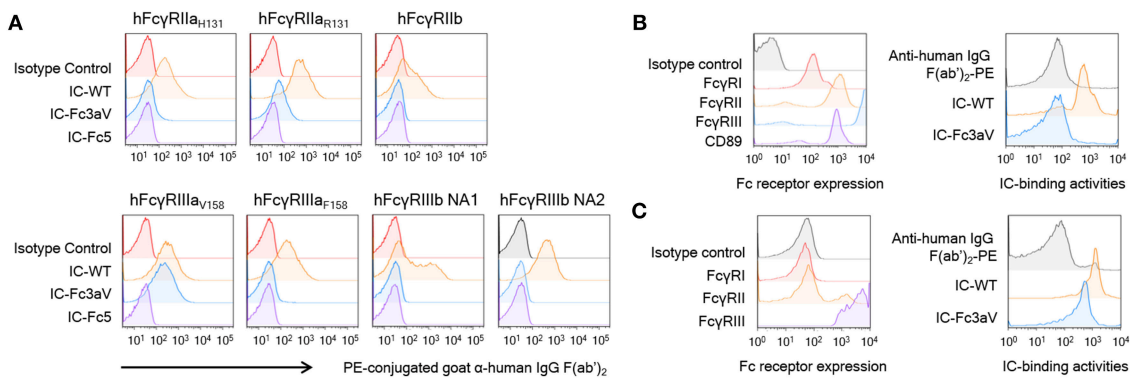

FIGURE 2 | Fc3aV binds to FcγRIIIA$_{V158}$ with exquisite selectivity **(A)** Binding activities of ICs (10 μg/ml), formed by mixing antibodies with F(ab′)$_2$ anti-hIgG F(ab′)$_2$-PE, to human FcγRs expressed by transfectant CHO cell measured by FACS, when antibodies are: isotype control (red), wt Trastuzumab (orange), Trastuzumab-Fc3aV (sky blue), and Trastuzumab-Fc5 (purple). **(B,C)** Binding activities of ICs on human neutrophils **(B)** or NK cells **(C)**; immune complexes were formed and used at the same concentration as in **(A)**. Left panels: Expression level of FcγRs on neutrophils activated by IFN-γ **(B)** or NK cells **(C)** detected by the respective anti-FcγR antibodies conjugated to APC; Right panels: Binding of ICs formed by wt or Fc3aV Trastuzumab, onto neutrophils or NK cells, respectively. Representative data of three independent experiments are shown.

competitor (**Figures 1A,B**). Ninety-six individual clones from the 4th round of sorting were randomly picked and analyzed further. The 96 clones encoded 22 unique hFc variants in which S239T, V264E, V282M, T299A, L309Q, and A378V amino acid mutations were enriched (**Figure S1**). Interestingly, 16 out of 22 clones contained a T299A substitution in the C'E loop of CH2 domain. The T299A mutation disrupts the canonical N-X-T/S N-linked glycosylation motif on the hFc and impairs, but not completely abolishes, hFcγR binding [(24), **Figure S3**]. Bacteria displaying IgGs with three different mutant Fc domains,

namely, TEMA (S239T, V264E, V282M, T299A), EAQ (V264E, T299A, L309Q), and Fc3aV (S239T, P248L, V264E, V282M, T299A, L309Q, A378V), were analyzed by FACS (**Figure S2**). Cells expressing TEMA, EAQ and Fc3aV Fc mutated IgGs showed 16-, 48.2-, and 62.4-fold increased binding activity for hFcγRIIIa$_{V158}$-GST dimers, respectively, compared with wild-type (wt) aglycosylated IgG1. In addition, TEMA and Fc3aV also slightly bound to hFcγRIIa or hFcγRIIb under these experimental conditions (**Figure S2**).

Several antibodies containing subsets of the amino acid substitutions found in TEMA, EAQ, and Fc3aV were expressed and analyzed in terms of their binding affinity and selectivity to purified monomeric, high-affinity hFcγRI-His or to GST-fused dimeric (and thus, higher avidity) versions of the low-affinity receptors, hFcγRIIa, hFcγRIIb, and hFcγRIIIa (36, 40). Three isolated Fc variants shared two mutations, V264E and T299A. First, the effect of V264E on the hFcγR-binding properties of aglycosylated Fc domain was investigated (**Figure S3**). Trastuzumab-V264E/T299A, showed higher hFcγRIIIa binding and decreased hFcγRIIb binding relative to the T299A variant (**Figure S3**). Similarly, Trastuzumab-EAQ (V264E, T299A, L309Q) showed higher affinity for hFcγRIIIa but lower hFcγRIIb binding relative to Trastuzumab-T299A/L309Q (**Figure S3**). Trastuzumab-Fc3aV showed selective binding to FcγRIIIa$_{V158}$ by ELISA, but still displayed some binding toward FcγRIIIa$_{F158}$ and FcγRIIb (**Figure S3**).

Due to the low sensitivity achieved by direct ELISA, we opted to measure the Fc-FcγR interactions in a highly sensitive SPR experiment. Trastuzumab-Fc3aV showed specific binding to GST-fused dimeric hFcγRIIIa$_{V158}$ ($K_D = 0.2 \pm 0.01\,\mu M$) without any significant binding to 400 nM of GST-fused dimeric hFcγRIIa$_{H131/R131}$, hFcγRIIb, and hFcγRIIIa$_{F158}$ by SPR (**Figure S4A** and **Table S1**). The equilibrium dissociation constants for the binding of Trastuzumab-Fc3aV to monomeric hFcγRs were also determined by SPR. Trastuzumab-Fc3aV exhibited no detectable response for any monomeric hFcγR except for hFcγRIIIa$_{V158}$ to which it bound with a $K_D = 4 \pm 1.0\,\mu M$ (**Figures 1C,D** and **Figure S4B,C**).

6The binding of ICs onto cells represents an exquisitely sensitive assay for detecting physiologically relevant IgG:FcγR interactions (14, 52). Large, high avidity ICs were formed by mixing Fc3aV or other antibodies with goat F(ab′)$_2$ anti-human F(ab′)$_2$ and binding to CHO cells expressing the low affinity hFcγRIIa$_{R131}$, hFcγRIIa$_{H131}$, hFcγRIIb, hFcγRIIIa$_{V158}$, and importantly hFcγRIIIa$_{F158}$ which only differs from hFcγRIIIa$_{V158}$ by one amino acid or the hFcγRIIIb-NA1 or hFcγRIIIb-NA2 allotype which differs from hFcγRIIIa by 6–8 amino acids at high levels was evaluated by FACS. IC formed by Trastuzumab Fc3aV bound to hFcγRIIIa$_{V158}$ expressing CHO cells but did not show significant binding activities for all other hFcγRs (**Figure 2A**). To examine whether ICs of Trastuzumab Fc3aV bind to hFcγRI-expressing cells in a physiologically relevant context we determined binding to neutrophils from human blood, which are activated with IFN-γ. As expected, ICs formed with Trastuzumab Fc3aV did not bind to activated neutrophils which express hFcγRI, hFcγRIIa/b, and hFcγRIIIb but not hFcγRIIIa (**Figure 2B**) (53).

hFcγRIIIa on the surface of NK cells has a different *N*-glycan composition relative to hFcγRIIIa on the surface of monocytes/macrophages or with hFcγRIIIa ectodomain produced recombinantly in Expi293F cells. Differences in receptor glycosylation can affect the affinity for IgG (54). Nonetheless, we found that, ICs formed with Trastuzumab Fc3aV show slightly lower binding to NK cells as those formed by wild-type antibodies, a finding entirely consistent with the lower affinity of Fc3aV determined by SPR (**Figures 1D**,**2C**).

hFcγRIIIa-Mediated Effector Functions in Various Effector Cells

To determine how hFcγRIIIa contributes to immune effector functions, we performed ADCP and ADCC assays using anti-CD20 Rituximab-Fc3aV or anti-Her2 Trastuzumab-Fc3aV and CD20$^+$ Raji cells or Her2^{high} SK-BR-3 breast cancer cells as targets, respectively. As expected, Fc3aV-formatted antibodies with Trastuzumab or Rituximab Fab showed equivalent binding with their wt counterparts to CD20$^+$ Raji cells or Her2^{high} SK-BR-3 breast cancer cells, respectively since mutations of the Fc domain did not affect antigen binding (**Figure S5**). We first performed ADCP assays with gmMϕ, which had been differentiated *in vitro* from human peripheral blood-derived CD14$^+$ monocytes. gmMϕ express hFcγRI at moderate, hFcγRIIa/b at high and hFcγRIIIa$_{V158}$ at low levels, as determined by flow cytometry (**Figure 3A**) (30–32, 35). Raji cells were labeled with fluorescent PKH67 and then opsonized with Fc-engineered Rituximab variants, followed by incubation with gmMϕ, as previously reported (40, 48). The extent of phagocytosis was evaluated using flow cytometry by determining the percentage of cells staining double positive for PKH67 and anti-CD14-APC or anti-CD11b-APC over the total number of PKH67$^+$ cancer cells. We found that Rituximab-Fc3aV, which as described above binds only to hFcγRIIIa$_{V158}$, has ADCP activity that is comparable to that of wt Rituximab (**Figures 3B,C**).

hFcγRIIIa-mediated phagocytosis by gmMϕ was also confirmed with Her2^{high} SK-BR-3 cancer cells opsonized with Trastuzumab-Fc3aV antibodies (**Figures 3B**,**D**). ADCP by either wt IgG- or Fc3aV- were significantly inhibited by the addition of a blocking, anti-CD16 F(ab′)$_2$ [mAb clone 3G8 (49–51)], confirming the importance of hFcγRIIIa in ADCP (**Figures 3C,D**). Opsonization of CD20$^+$ Raji cells with Rituximab equipped with Fc5, an engineered hFc domain which as discussed above engages hFcγRI selectively, resulted in a low, but statistically significant level of ADCP. However, we did not detect phagocytosis of Her2$^+$ SK-BR-3 cells using Trastuzumab-Fc5 (**Figures 3C,D**).

We also determined ADCC activity by detecting calcein-AM release from cancer cells (CD20$^+$ Raji cells) with PBMC, monocytes, or NK cells, isolated from the same healthy donor (V/V or V/F genotype), as effectors ($n = 3$ donors). Based on the weaker binding activity of immune complexes with Fc3aV antibodies on NK cells compared to that of w.t. IgG1 antibodies (**Figure 2C**), the ADCC potency of PBMCs, or NK cells using Rituximab-Fc3aV was weaker than the level observed with wt glycosylated IgG1 Rituximab (**Figures 4A,B**). Similarly, Rituximab-Fc3aV showed significant ADCC activity

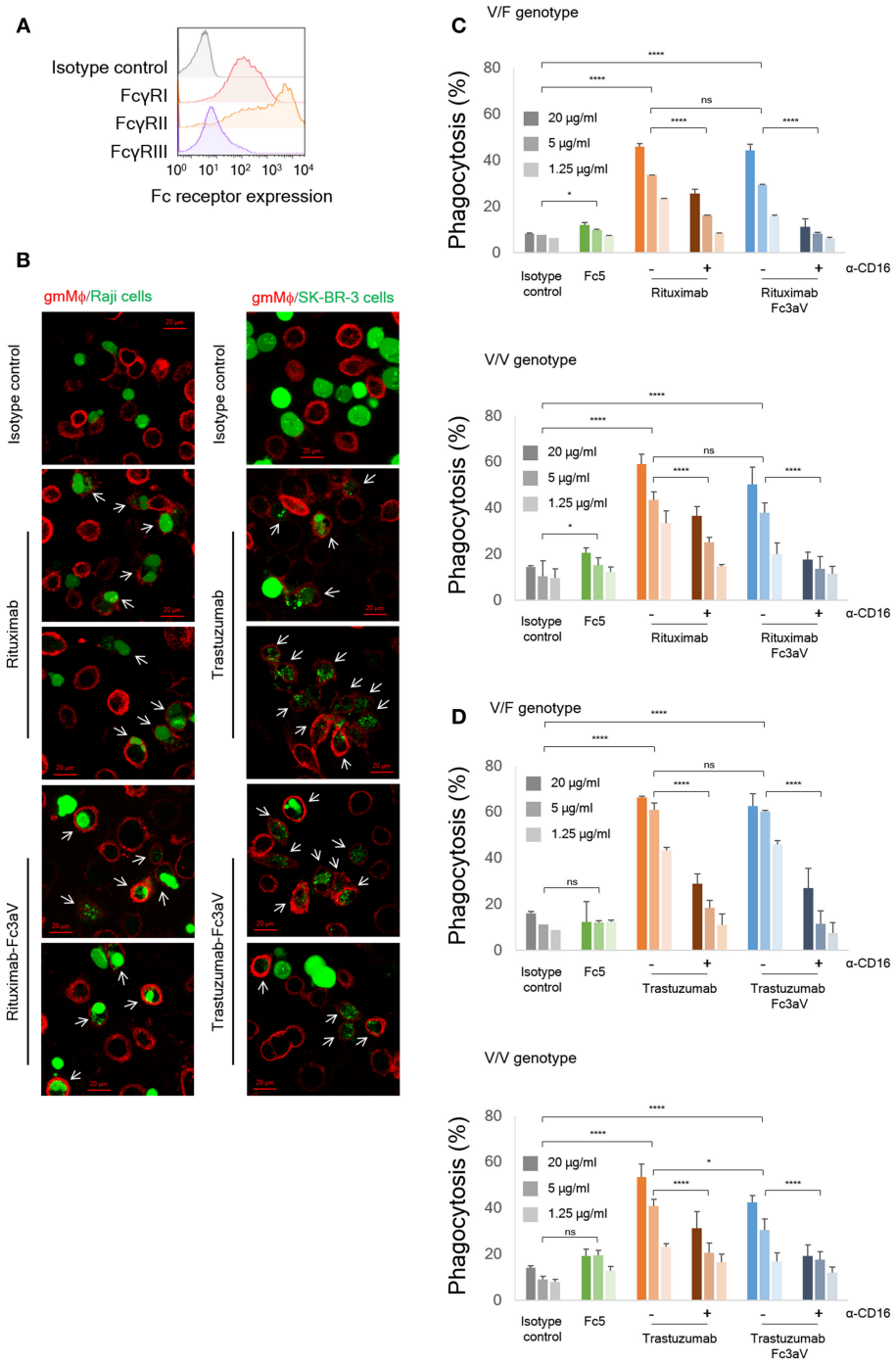

FIGURE 3 | *In vitro* phagocytosis assays. **(A)** Expression levels of FcγRs on monocyte-derived human gmMϕ were detected by FACS using the respective anti-FcγR-APC antibodies. **(B)** Fluorescent images of phagocytosis of cancer cells (green, left panel: Raji and right panel: SK-BR-3) by gmMϕ (red). Macrophages were stained with anti-CD14 and anti-CD11b-APC and cancer cells were stained with Calcein-AM. White arrows indicate phagocytosed cancer cells. **(C,D)** ADCP assays with gmMϕ (V/F and V/V genotypes) as effectors and **(C)** PKH67-labeled CD20^{+} Raji cells or **(D)** PKH67-labeled Her2^{high} SK-BR-3 cells as targets (Effector:Target ratio of 10:1). Macrophages were pre-incubated with or without 10 µg/ml of anti-CD16 mAb 3G8 F(ab′)$_2$ for 10 min prior to the addition of wt or variants of trastuzumab. ADCP data are shown for an FcγRIIIa heterozygous donor (V/F) and one donor homozygous for the FcγRIIIa$_{V158}$ allotype (V/V). Representative data from for independent experiments. Error bars are standard deviations of triplicate samples. Statistical analysis was performed by two way ANOVA with Tukey's multiple comparisons test (ns: $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

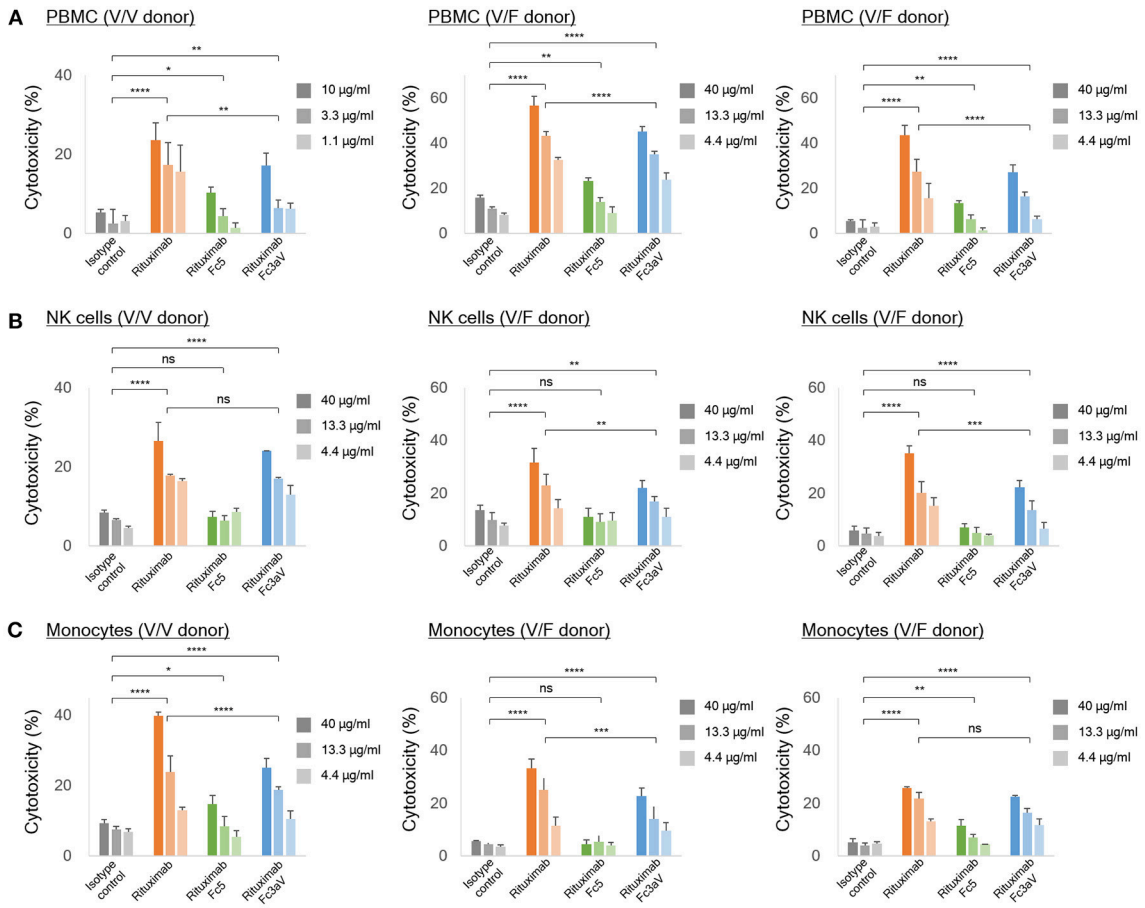

FIGURE 4 | *In vitro* ADCC assays. ADCC assays with calcein-AM loaded Raji cells as targets and **(A)** PBMCs, **(B)** NK cells, and **(C)** freshly isolated monocytes from the same healthy donor [homozygous V/V (left) or heterozygous V/F (center and right) and E:T ratio = 10:1] as effector cells. **(A–C)** The level of the released calcein-AM was measured to detect target cell lysis after 4 h. Error bars correspond to standard deviations of triplicate samples. Statistical analysis was performed by two way ANOVA with Tukey's multiple comparisons test (ns: $P > 0.05$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$).

with monocytes from one V/V and two V/F donors, which was weaker than w.t. Rituximab (**Figure 4C**). Consistent with our findings, recently Yeap et al. presented evidence that hFcγRIIIa (CD16a) ligation is important for ADCC by monocytes. We also examined whether antibodies formatted with the hFcγRI-specific Fc5 can trigger ADCC. Rituximab-Fc5 initiated a low but statistically significant activity of ADCC with PBMCs as effectors in all donors analyzed (**Figure 4A**). With monocytes we only observed a low level of hFcγRI mediated ADCC in two out of three donors, consistent with the high degree of hFcγRI variability from donor to donor (**Figure 4C**). Finally, as expected, Fc5 formatted antibodies did not trigger ADCC with NK cells which do not express any hFcγRI (**Figure 4B**).

DISCUSSION

A number of engineered Fc domains including the SDIE (S239D/ I332E), SDALIE (S239D/ A330L/ I332E), S298A/ E333A/ K334A, GASDALIE (G236A/ S239D/ A330L/ I332E), and GASDIE (G236A/S239D/I332E) variants showing increased hFcγRIIIa affinity had been engineered (5, 55). However, all these Fcs show detectable and, in some instances, high affinity to other hFcγR and therefore they are not suitable for mechanistic studies to selectively delineate the phenotypes induced solely via the engagement of FcγRIIIa. Removal of the single glycan in the IgG hFc domain markedly increases the flexibility of the CH2 conformation resulting in dramatically decreased binding to all hFcγRs as well as to C1q (44). We had shown earlier that mutations in aglycosylated human IgG Fc domains can stabilize a particular CH2 conformer and restore binding selectively either to a single Fc binding protein, e.g., hFcγRI or C1q or, alternatively to multiple hFcγRs (36, 39, 40). Extending these earlier studies, we have now engineered an aglycosylated hFc variant, Fc3aV, with high selectivity for hFcγRIIIa$_{V158}$ and containing seven amino acids substitutions (S239T, P248L, V264E, V282M, T299A, L309Q, and A378V). Antibodies with the Fc3aV hFc domain do not show any binding to the high affinity receptor hFcγRI or to dimeric high avidity versions of the low affinity receptors hFcγRIIa/b by SPR. Consistent with this biophysical data, ICs formed by antibodies formatted

with the Fc3aV domain did not bind significantly to CHO-cells expressing low-affinity hFcγRs except hFcγRIIIa$_{V158}$. Since hFcγRIIIa glycoproteins expressed by HEK293 or CHO cells are exclusively modified by biantennary complex-type glycans with partial sialylation (56), and the *N*-glycan composition can in turn affect its affinity for IgG (54, 57) we also evaluated binding activity of ICs to primary cells. Furthermore, immune complexes of Fc3aV antibodies bind to NK cells which express hFcγRIIIa and not to activated neutrophils which express hFcγRI, hFcγRIIa, and hFcγRIIIb but not hFcγRIIIa (**Figures 2B,C**).

We used Fc3aV to clarify the contribution of hFcγRIIIa to ADCP. Data from clinical studies and animal models have underscored the significance of phagocytosis in the clearance of cancer cells (7, 25–29, 58). Importantly, the depletion of macrophages in mouse models was shown to abolish the therapeutic efficacy of anti-CD20, anti-CD30, or anti-CD40 antibodies in B-cell cancer models, in contrast to depletion of NK cells or neutrophils (26–29).

While earlier reports had suggested that ADCP is mediated primarily by binding to hFcγRIIa (37) here we present evidence showing that selective engagement of human hFcγRIIIa, is sufficient to trigger potent phagocytosis by gmMϕ. The ADCP activity we observed with CD20$^+$ or Her2$^+$ cancer cells as targets and gmMϕ as effectors is particularly noteworthy because, as had been reported earlier and confirmed here, cell surface expression of hFcγRIIIa on gmMϕ is significantly lower than that of hFcγRIIa and FcγRI [**Figure 3A**,(36–38)]. One reason why selective ligation of hFcγRIIIa by Fc3aV antibodies results in ADCP that is comparable to that observed with wt Rituximab which binds to all hFcγR on macrophages and notably the much more highly expressed hFcγRIIa is that, unlike wt antibodies Fc3aV does not bind to the inhibitory hFcγRIIb and therefore is unable to trigger ITIM phosphorylation and downstream signaling processes. hFcγRIIb ligation is known to inhibit the effector functions initiated by the engagement of the activating Fc receptors including ADCP (2). We note that human macrophages show a large degree of phenotypic variations, which depend on the tissue of origin and on culture method *in vitro* and is associated with significant differences in FcγR expression levels and especially hFcγRIIIa. For example, red pulp macrophages express significantly higher levels of FcγRIIIa relative to the other FcγRs (31). Similarly *in vitro* differentiated macrophages stimulated with M-CSF have generally higher levels of hFcγRIIIa compared to gmMϕ (59, 60). Moreover, donor to donor variation effects in ADCP assays are routinely observed. Along these lines, we note that the data in **Figure 3D** shows that with Trastuzumab-Fc3aV antibodies (but not when using Rituximab-Fc3aV and Raji cells as targets, **Figure 3C**), the absolute level of phagocytosis in an hFcγRIIIa V/F heterozygous donor was slightly higher than that observed in a different donor expressing the V/V allele. The fact that high ADCP, at a level comparable to that observed with w.t. antibodies, could be mediated with the Fc3aV variant in four donors, with different target cells (Raji or SK-BR-3) and furthermore, that phagocytosis by Fc3aV antibodies was inhibited by hFcγRIIIa-blocking antibodies support our conclusion that hFcγRIIIa engagement alone is sufficient to trigger significant ADCP by gmMϕ.?NK cells which only express hFcγRIIIa and can release cytotoxic granules are considered to be the most important contributor to ADCC *in vitro* with PBMCs as effector cells (2). As expected, opsonization of tumor cells with Fc3aV antibodies triggered potent ADCC with both PBMCs and with NK cells as effectors. Similarly we found that the selective ligation of hFcγRIIIa on monocytes strongly triggers ADCC, a finding consistent with recent reports (10). As with the ADCP results discussed above, donor to donor variability precludes a comparison of subtle differences in the absolute magnitude of ADCC among donors. For example, in **Figure 4**, with PBMCs but not with monocytes or NK cells as effectors, the level of ADCC in a donor that is homozygous for the expression of the high affinity 158 V allele was lower than that of the V/F heterozygous donors. This may be in part due to the fact that the level of ADCC with PBMCs as effectors depends on factors other than the hFcγRIIIa allotype including, importantly, the ratio and phenotypes of monocytes to NK cells, since cytokines or receptors expressed by monocytes can impact NK cell-mediated cytotoxicity (61).

Somewhat unexpectedly we found that antibodies with the hFcγRI-selective Fc5 domain elicited much weaker ADCP with gmMϕ opsonized with Fc3aV antibodies, even though as shown in **Figure 3A** FACS analysis shows a >10-fold lower signal for hFcγRIIIa on gmMϕs compared that for hFcγRI. However, it should be noted that the FACS data in **Figure 3A** does not reflect the absolute levels of the different receptors since the FACS data were obtained with different antibodies having different affinities for their respective hFcγR antigens. This effect is not due to poor activation of hFcγRI by Fc5 antibodies as we had established earlier that Fc5 antibodies are efficient in activating hFcγRI and initiating target cell clearance by classical dendritic cells (39). The weaker effector functions associated with ligation of hFcγRI may be due to subtle, cell-specific effects related to differences in downstream signaling following ITAM phosphorylation and the elucidation of these effects will require further investigation.

In summary, functional studies with macrophages and other myeloid-derived cells capable of clearing opsonized targets using Fc3aV formatted antibodies may prove very useful for elucidating the role of hFcγRIIIa ligation on the importance of different effector functions and leukocytes subsets for the mechanism of action of therapeutic antibodies. Myeloid cells express multiple hFcγRs and therefore when they are activated with immune complexes formed by numerous antibodies the resulting effector functions represent the integrated effect due to signaling by all the hFcγRs. The use of engineered Fc domains with absolute selectivity for only one hFcγR can be a powerful tool for elucidating the subtle signaling differences and phenotypic effects elicited by each individual Fc receptor and how these ultimately contribute to the clearance of target pathogenic cells.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

TK, C-HL, and GG conceived and designed the research. C-HL, TK, GD, JJ, OG, JL, JK, and WC performed the experiments. GG, C-HL, TK, GD, JJ, and PB analyzed the data. GG, C-HL, TK, GD, JJ, JL, and PB wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00562/full#supplementary-material

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Parameter Identification for a Model of Neonatal Fc Receptor-Mediated Recycling of Endogenous Immunoglobulin G in Humans

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Salvage of endogenous immunoglobulin G (IgG) by the neonatal Fc receptor (FcRn) is implicated in many clinical areas, including therapeutic monoclonal antibody kinetics, patient monitoring in IgG multiple myeloma, and antibody-mediated transplant rejection. There is a clear clinical need for a fully parameterized model of FcRn-mediated recycling of endogenous IgG to allow for predictive modeling, with the potential for optimizing therapeutic regimens for better patient outcomes. In this paper we study a mechanism-based model incorporating nonlinear FcRn-IgG binding kinetics. The aim of this study is to determine whether parameter values can be estimated using the limited *in vivo* human data, available in the literature, from studies of the kinetics of radiolabeled IgG in humans. We derive mathematical descriptions of the experimental observations—timecourse data and fractional catabolic rate (FCR) data—based on the underlying physiological model. Structural identifiability analyses are performed to determine which, if any, of the parameters are unique with respect to the observations. Structurally identifiable parameters are then estimated from the data. It is found that parameter values estimated from timecourse data are not robust, suggesting that the model complexity is not supported by the available data. Based upon the structural identifiability analyses, a new expression for the FCR is derived. This expression is fitted to the FCR data to estimate unknown parameter values. Using these parameter estimates, the plasma IgG response is simulated under clinical conditions. Finally a suggestion is made for a reduced-order model based upon the newly derived expression for the FCR. The reduced-order model is used to predict the plasma IgG response, which is compared with the original four-compartment model, showing good agreement. This paper shows how techniques for compartmental model analysis—structural identifiability analysis, linearization, and reparameterization—can be used to ensure robust parameter identification.

Keywords: biological systems, lumped-parameter systems, immunoglobulin G, neonatal Fc receptor, parameter estimation, structural identifiability

1. INTRODUCTION

Immunoglobulin G (IgG) is the most abundant immunoglobulin (Ig) isotype in the circulation in humans, with a plasma concentration in healthy adults of 10–16 $g\,l^{-1}$ (1). Its high concentration is facilitated by the neonatal Fc receptor (FcRn), which binds IgG in intracellular endosomes and transports it to the plasma membrane to be returned to the circulation. A proportion of IgG molecules that are not bound by FcRn are degraded in lysosomes. In this way, FcRn continually protects a proportion of the circulating IgG from degradation. The recycling mechanism is saturable, such that at high plasma IgG concentrations a greater proportion of plasma IgG is degraded. Conversely, at depleted plasma IgG concentrations, a greater proportion is recycled and the half-life is extended beyond the normal 23 days (2).

Recent publications have drawn attention to the importance of FcRn-mediated recycling of endogenous IgG in the bone marrow cancer multiple myeloma. In multiple myeloma, clonal plasma cells secrete an excess of monoclonal Ig into the circulation. Patients undergoing therapy are primarily monitored by quantification of Ig in blood serum samples (3). Mills et al. (4) have suggested that FcRn-mediated recycling of IgG may result in different response rates between patients with IgG-producing multiple myeloma and patients with IgA-producing multiple myeloma. Yan et al. (5) have also suggested that FcRn-mediated recycling of endogenous IgG in patients with multiple myeloma may shorten the half-life of the therapeutic monoclonal antibody daratumumab. These studies highlight the need for a parameterized model of endogenous IgG kinetics for investigating these clinical scenarios.

Numerous mathematical models of IgG kinetics have been presented in the literature, mostly with the aim of describing the pharmacokinetics of therapeutic monoclonal antibodies (mAbs) that are also regulated by FcRn. Many of these models are therefore pharmacokinetic in nature: their parameter values are obtained from animal experiments and they may be physiologically-based, with up to around 10 organs explicitly represented in the model (6–14). Pharmacokinetic models developed for specific mAbs may not be generalizable to endogenous IgG if, for example, they include details such as binding of the mAb to its target. In addition, mAb disposition may be adequately described by linear models in many cases where the plasma concentration of therapeutic mAb is substantially smaller than the plasma concentration of endogenous IgG and the latter is constant (13, 14). However, the assumption of a constant plasma concentration of IgG is not always appropriate; for example, in multiple myeloma the plasma IgG concentration typically shows large changes during the course of therapy. Relative to a less complex model, the more complex model will usually provide a better fit to observed data. However, this alone does not imply that all the parameters in the complex model can be estimated consistently, nor does it imply that the underlying assumptions of the complex model are valid (15).

In this paper we study a mechanism-based model with a single plasma compartment, rather than separate plasma compartments for different organs, which is accessible to measurement in humans. The model, which has been previously shown by Kim et al. (16) and Hattersley (17), has in total four compartments, representing IgG in plasma, IgG in a peripheral compartment (representing less rapidly perfused tissues), unbound IgG in intracellular endosomes and IgG bound to FcRn receptors in intracellular endosomes. The IgG-FcRn interaction is represented by nonlinear receptor-ligand binding kinetics (6, 7, 9). With reliable parameter values for humans, it may be possible to use this model to predict the responses of plasma IgG under various clinical conditions.

The aim of this study is to determine whether the model parameter values can be obtained using the limited *in vivo* human data that are available in the literature. The data are from studies of the kinetics of administered small doses of radiolabeled IgG when the subject's endogenous IgG is in steady state. We consider two measured outputs: the timecourse of the proportion of an administered dose of radiolabeled IgG remaining in plasma and in the body; and the relationship between the fractional catabolic rate and the quantity of endogenous IgG in plasma. Structural identifiability analysis is performed with respect to these outputs and structurally identifiable parameters are estimated from the data.

2. MATHEMATICAL MODELS AND DATA DESCRIPTION

2.1. The Four Compartment Model

The model of IgG metabolism under study (16, 17) has four state variables, nine parameters, and an input function, $I(t)$, representing the synthesis of IgG. The model equations are given by

$$\begin{aligned}
\dot{x}_1(t) &= -(k_{21}+k_{31})x_1(t) + k_{12}x_2(t) + k_{14}x_4(t) + I(t)\\
\dot{x}_2(t) &= k_{21}x_1(t) - k_{12}x_2(t)\\
\dot{x}_3(t) &= k_{31}x_1(t) - k_{03}x_3(t) - \frac{k_{\text{on}}}{v_3}x_3(t)(R_{\text{tot}} - x_4(t)) + k_{\text{off}}x_4(t)\\
\dot{x}_4(t) &= \frac{k_{\text{on}}}{v_3}x_3(t)(R_{\text{tot}} - x_4(t)) - (k_{14}+k_{\text{off}})x_4(t),
\end{aligned} \tag{1}$$

where $x_1(t)$, $x_2(t)$, $x_3(t)$, and $x_4(t)$ represent the quantities in μmol of IgG in plasma, IgG in a peripheral compartment, unbound IgG in endosomes and IgG bound to FcRn in endosomes, respectively. $I(t)$ represents the rate of synthesis of IgG in $\mu\text{mol}\,\text{day}^{-1}$. The rate constants, k_{ij}, represent the rate of material flow from compartment j to compartment i, with the convention that 0 represents the environment outside the system. k_{on} and k_{off} are the receptor-ligand binding constants of IgG and FcRn. We denote the volumes of plasma, the peripheral compartment and the endosomes by v_1, v_2, and v_3, respectively. We assume a constant total (bound and unbound) quantity of FcRn, R_{tot} (6). This means that the quantity of unbound FcRn is represented by $[R_{\text{tot}} - x_4(t)]$. The state variables of the model and physiological interpretations of the parameters are summarized in **Table 1**. Note that all states and parameters can only take non-negative values. We refer to **Figure 1** for a schematic of the model.

TABLE 1 | States and parameters of four-compartment model of IgG metabolism, with parameter values sourced in the literature.

Name	Units	Literature value	Physiological interpretation
x_1	μmol	–	Quantity of IgG in the central (plasma) compartment
x_2	μmol	–	Quantity of IgG in the peripheral compartment
x_3	μmol	–	Quantity of unbound IgG in intracellular endosomes
x_4	μmol	–	Quantity of IgG-FcRn complexes in intracellular endosomes
v_1	l	2.9*	Plasma volume
v_2	l	–	Volume of peripheral compartment
v_3	l	0.34†	Total volume of endosomes
k_{21}	day^{-1}	0.51‡	Rate constant of flow of IgG from plasma to peripheral compartment
k_{31}	day^{-1}	0.18§	Rate constant of flow of IgG from plasma into endosomes by pinocytosis
k_{12}	day^{-1}	0.41‡	Rate constant of flow of IgG from peripheral compartment to plasma
k_{14}	day^{-1}	5.0¶	Rate constant of flow of recycled IgG from endosomes back into plasma
k_{03}	day^{-1}	3.0‖	Rate constant of degradation of unbound IgG in endosomes
k_{on}	$l\mu mol\,day^{-1}$	1,000**	Association rate constant of IgG-FcRn binding
R_{tot}	μmol	14¶	Total quantity of FcRn receptors, bound and unbound
k_{off}	day^{-1}	100**	Dissociation rate constant of IgG-FcRn binding

*Solomon et al. (18), †Shah and Betts (19), ‡Hattersley et al. (20), §Waldmann and Strober (21), ¶Ferl et al. (6), ‖ Hansen and Balthasar (22), **Chen and Balthasar (10).

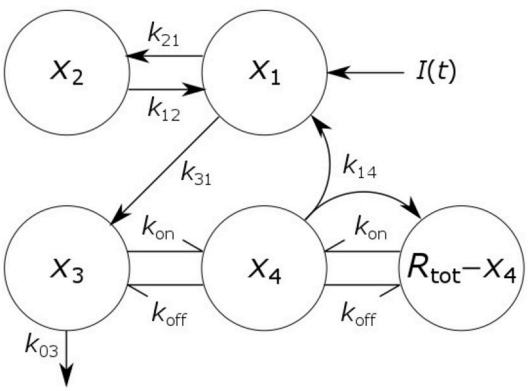

FIGURE 1 | Schematic of four-compartment model of IgG metabolism. Arrows represent material flow between compartments and hooked arrows represent nonlinear receptor-ligand binding. The fifth compartment shown ($R_{tot} - x_4$) represents unbound FcRn receptors and has been eliminated from the model equations. The arrow, labeled k_{14}, from the IgG-FcRn complex compartment (x_4) to the unbound FcRn receptor compartment ($R_{tot} - x_4$), represents internalization of FcRn receptors from the plasma membrane to the endosome, after releasing IgG.

When the production rate of IgG is constant, $I(t) = I_0$, the system has a stable equilibrium point given by

$$\begin{aligned}
\hat{x}_1 &= \frac{I_0\left(k_{03}k_{14}v_3 + k_{03}k_{off}v_3 + k_{on}I_0 + k_{14}k_{on}R_{tot}\right)}{k_{31}\left(k_{03}v_3(k_{14}+k_{off}) + k_{on}I_0\right)} \\
\hat{x}_2 &= \frac{k_{21}}{k_{12}}\hat{x}_1 \\
\hat{x}_3 &= \frac{I_0}{k_{03}} \\
\hat{x}_4 &= \frac{k_{on}I_0R_{tot}}{k_{03}v_3(k_{14}+k_{off}) + k_{on}I_0}.
\end{aligned} \tag{2}$$

A stability analysis for this equilibrium point is provided in the **Supplementary Material**.

2.2. *In vivo* Human Data From the Literature

The data available in the literature were obtained from tracer experiments. These studies entailed intravenous administration of a bolus dose of radiolabeled IgG (the tracer) and monitoring the proportion of the dose remaining in the blood and in the body over time. In this way the administered dose is distinguishable (by the experimenter) from the subject's own endogenous IgG. The quantity of administered tracer is small, so as not to perturb the steady state of the endogenous IgG. The purpose of tracer experiments is to enable observation of processes such as distribution and elimination undergone by the endogenous protein, whilst it is in steady state. The methods are described fully by Waldmann and Strober (21).

The data for an individual subject consist of the timecourse of the proportion of the injected dose of IgG remaining in plasma and the timecourse of the proportion of dose remaining in the body. In this paper we use the data from six such plots available in the literature. We refer to the individuals as subjects A–F. The timecourse data for subjects A–D are from Solomon et al. (18), for subject E from Waldmann and Terry (23), and for subject F from Waldmann and Strober (21). Several of the individuals have health conditions which may result in an increased or decreased plasma IgG concentration. Subjects A and C have IgG multiple myeloma and subject D has macroglobulinemia. Subjects B, E, and F are referred to as "normal" subjects. A spaghetti plot of the data is shown in **Figure 2A**. Subjects A and D show slower dynamics and subject C shows faster dynamics. The dynamics of IgG in these subjects is assumed to be described by the same model, as given by Equations (1), however they may have had altered production rates of IgG due to the diseases.

Also available in the literature is a plot of the fractional catabolic rate (FCR) vs. the subject's plasma concentration of endogenous IgG, obtained from a group of individuals with a

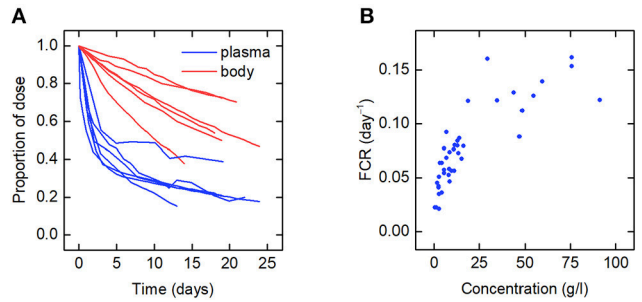

FIGURE 2 | (A) Spaghetti plot of the proportion of administered IgG remaining in plasma (blue) and the body (red) in six subjects; data from Waldmann and Strober (21), Solomon et al. (18), and Waldmann and Terry (23). **(B)** Plasma concentration dependence of the fractional catabolic rate (FCR); redrawn from Waldmann and Strober (21), with permission from S. Karger AG, Basel.

range of plasma IgG concentrations (21). The FCR is defined as the elimination rate of IgG as a fraction of the quantity of IgG in plasma. In practice the FCR is calculated from the rate at which the tracer dose leaves the body at time t divided by the proportion of tracer dose remaining in plasma at time t. The relationship between the FCR and the timecourse data is described further in section 2.6. A plot of the FCR vs. the plasma concentration of endogenous IgG for 41 individuals provided by Waldmann and Strober (21) is shown in **Figure 2B**. Each data point was derived from the timecourse data of an individual subject. All of the data described in this section were extracted from plots in the literature using the Digitizer tool in OriginPro 2016 (24).

2.3. Nonlinear Model of Coupled Tracer and Endogenous IgG Dynamics

The administered tracer and the endogenous IgG are assumed to be indistinguishable by the human body, that is they exhibit identical kinetic (input/output) behavior—a standard assumption in tracer studies (25). We therefore assume that the kinetics of both tracer and endogenous IgG are described by the model given by Equations (1). From Equations (1), letting $x_i(t) = x_{i,\mathrm{T}}(t) + x_{i,\mathrm{E}}(t)$, where $x_{i,\mathrm{T}}(t)$ and $x_{i,\mathrm{E}}(t)$ denote the quantities in μmol in compartment i of radiolabeled and endogenous IgG, respectively, gives

$$
\begin{aligned}
\dot{x}_{1,\mathrm{T}}(t) &= -(k_{21}+k_{31})x_{1,\mathrm{T}}(t) + k_{12}x_{2,\mathrm{T}}(t) + k_{14}x_{4,\mathrm{T}}(t)\\
\dot{x}_{2,\mathrm{T}}(t) &= k_{21}x_{1,\mathrm{T}}(t) - k_{12}x_{2,\mathrm{T}}(t)\\
\dot{x}_{3,\mathrm{T}}(t) &= k_{31}x_{1,\mathrm{T}}(t) - k_{03}x_{3,\mathrm{T}}(t) - \frac{k_{\mathrm{on}}}{v_3}x_{3,\mathrm{T}}(t)(R_{\mathrm{tot}} - x_{4,\mathrm{E}}(t)\\
&\quad - x_{4,\mathrm{T}}(t)) + k_{\mathrm{off}}x_{4,\mathrm{T}}(t)\\
\dot{x}_{4,\mathrm{T}}(t) &= \frac{k_{\mathrm{on}}}{v_3}x_{3,\mathrm{T}}(t)(R_{\mathrm{tot}} - x_{4,\mathrm{E}}(t) - x_{4,\mathrm{T}}(t)) - (k_{14}+k_{\mathrm{off}})x_{4,\mathrm{T}}(t)\\
\dot{x}_{1,\mathrm{E}}(t) &= -(k_{21}+k_{31})x_{1,\mathrm{E}}(t) + k_{12}x_{2,\mathrm{E}}(t) + k_{14}x_{4,\mathrm{E}}(t) + I_{\mathrm{E}}\\
\dot{x}_{2,\mathrm{E}}(t) &= k_{21}x_{1,\mathrm{E}}(t) - k_{12}x_{2,\mathrm{E}}(t)\\
\dot{x}_{3,\mathrm{E}}(t) &= k_{31}x_{1,\mathrm{E}}(t) - k_{03}x_{3,\mathrm{E}}(t) - \frac{k_{\mathrm{on}}}{v_3}x_{3,\mathrm{E}}(t)(R_{\mathrm{tot}} - x_{4,\mathrm{E}}(t)\\
&\quad - x_{4,\mathrm{T}}(t)) + k_{\mathrm{off}}x_{4,\mathrm{E}}(t)\\
\dot{x}_{4,\mathrm{E}}(t) &= \frac{k_{\mathrm{on}}}{v_3}x_{3,\mathrm{E}}(t)(R_{\mathrm{tot}} - x_{4,\mathrm{E}}(t) - x_{4,\mathrm{T}}(t))\\
&\quad - (k_{14}+k_{\mathrm{off}})x_{4,\mathrm{E}}(t).
\end{aligned}
\tag{3}
$$

I_{E} (μmol day^{-1}) represents the production rate of endogenous IgG, which is assumed constant. All other parameters are defined in **Table 1**.

The dose of tracer administered at time $t = 0$ days is treated as a non-zero initial condition for $x_{1,\mathrm{T}}(t)$. Tracer is administered to the plasma compartment only; therefore the initial conditions of the remaining tracer compartments are zero. The endogenous IgG is assumed to be in steady state throughout the experiment, such that the initial conditions of the endogenous IgG are given by the steady states in Equations (2), with $I_0 = I_{\mathrm{E}}$. In summary, the initial conditions are given by

$$
\begin{aligned}
x_{1,\mathrm{T}}(0) &= D\\
x_{2,\mathrm{T}}(0) &= x_{3,\mathrm{T}}(0) = x_{4,\mathrm{T}}(0) = 0\\
x_{1,\mathrm{E}}(0) &= \hat{x}_1\\
x_{2,\mathrm{E}}(0) &= \hat{x}_2\\
x_{3,\mathrm{E}}(0) &= \hat{x}_3\\
x_{4,\mathrm{E}}(0) &= \hat{x}_4,
\end{aligned}
\tag{4}
$$

where $\hat{x}_i$ is the steady state quantity of endogenous IgG in compartment i, given by Equations (2), and D (μmol) is the administered dose of tracer.

The experimenter observes the proportion of the dose remaining in plasma [denoted by $y_1(t)$] and in the body [denoted by $y_2(t)$] during the experiment. The observation functions are thus given by

$$
\begin{aligned}
y_1(t) &= \frac{x_{1,\mathrm{T}}(t)}{D}\\
y_2(t) &= \frac{x_{1,\mathrm{T}}(t) + x_{2,\mathrm{T}}(t) + x_{3,\mathrm{T}}(t) + x_{4,\mathrm{T}}(t)}{D}.
\end{aligned}
\tag{5}
$$

2.4. Linearized Model of Tracer Dynamics

Provided that the administered dose of tracer is sufficiently small, the tracer kinetics can be approximated using the Taylor series expansion of the model state about the equilibrium point. In this way a linear model of the experiment, valid in a neighborhood of the equilibrium point, is derived. Our derivation is provided in the **Supplementary Material**. The derivation of a linearized model for tracer dynamics from a general compartmental model is provided by Anderson (26).

The linear equations describing the tracer kinetics are given by

$$
\begin{aligned}
\dot{x}_{1,\mathrm{T}}(t) &= -(k_{21}+k_{31})x_{1,\mathrm{T}}(t) + k_{12}x_{2,\mathrm{T}}(t) + k_{14}x_{4,\mathrm{T}}(t)\\
\dot{x}_{2,\mathrm{T}}(t) &= k_{21}x_{1,\mathrm{T}}(t) - k_{12}x_{2,\mathrm{T}}(t)\\
\dot{x}_{3,\mathrm{T}}(t) &= k_{31}x_{1,\mathrm{T}}(t) - k_{03}x_{3,\mathrm{T}}(t) - k_{43}x_{3,\mathrm{T}}(t) + k_{34}x_{4,\mathrm{T}}(t)\\
\dot{x}_{4,\mathrm{T}}(t) &= k_{43}x_{3,\mathrm{T}}(t) - (k_{14}+k_{34})x_{4,\mathrm{T}}(t)
\end{aligned}
\tag{6}
$$

where $x_{1,\mathrm{T}}(t)$, $x_{2,\mathrm{T}}(t)$, $x_{3,\mathrm{T}}(t)$, and $x_{4,\mathrm{T}}(t)$ represent the quantities of radiolabeled IgG in the central compartment, in the peripheral compartment, unbound in intracellular endosomes, and bound

to FcRn in intracellular endosomes, respectively. The new parameters k_{34} and k_{43} are given by

$$k_{34} = k_{\text{off}}$$
$$k_{43} = \frac{k_{\text{on}}(R_{\text{tot}} - \hat{x}_4)}{v_3} = \frac{k_{\text{on}} R_{\text{tot}} k_{03}(k_{14} + k_{\text{off}})}{I_{\text{E}} k_{\text{on}} + k_{03} v_3 (k_{14} + k_{\text{off}})}. \quad (7)$$

All other parameters are defined in **Table 1**. The initial conditions are given by the first two equations of Equations (4) and the observation functions are given by Equations (5).

2.5. Comparison of Nonlinear Model and Linearized Model for Large Tracer Doses

The linearization of the model of timecourse observations relies on the assumption of a sufficiently small dose of tracer, such that the endogenous IgG can be assumed to remain in steady state. A typical tracer dose is between $3 \cdot 10^{-3}$ and $7 \cdot 10^{-3}$ μmol (18). Simulations of the quantity of tracer in each compartment are shown in **Figure 3**. In **Figure 3A**, a dose of $D = 1$ μmol is assumed and in **Figure 3B**, a dose of $D = 100$ μmol is assumed. The value of 1 μmol was chosen to show that the linear model is a valid approximation of the nonlinear model, even when the dose is more than 100 times typical tracer doses. The extremely large value of 100 μmol was chosen specifically to show the dynamics of the linearized model when it is not a valid approximation of the nonlinear model. The parameter values in **Table 1** are used. A normal IgG synthesis rate of $I_{\text{E}} = 15\,\mu\text{mol day}^{-1}$ was used; however the linearized model was still valid for $D = 1$ μmol when comparatively very small values of I_{E} were used. We find that, for a dose of 1 μmol and the particular parameter values used, the linearized model is a valid approximation of the full nonlinear model over a 25-day simulated time course. When the dose is increased to 100 μmol, the assumption that the steady state is not perturbed by the administered dose no longer holds and the two models give different simulation results for the quantities of tracer.

2.6. Fractional Catabolic Rate

We recall that the FCR (μmol day^{-1}) is defined as the elimination rate of IgG as a fraction of the quantity of IgG in plasma and can be defined with respect to the tracer or with respect to the endogenous IgG. The FCR with respect to the tracer is therefore given by

$$\text{FCR}_{\text{T}}(t) = \frac{k_{03} x_{3,\text{T}}(t)}{x_{1,\text{T}}(t)}, \quad (8)$$

where $x_{3,\text{T}}(t)$ and $x_{1,\text{T}}(t)$ are given by the solution of Equations (6).

Whilst a single value of the FCR is measured for an individual subject (see **Figure 2B**), in actuality $\text{FCR}_{\text{T}}(t)$ is not constant, as shown by the dependence on time in Equation (8). A simulation of $\text{FCR}_{\text{T}}(t)$ during the experiment is shown in **Figure 4**. After around day 5, for the particular parameter values used, $\text{FCR}_{\text{T}}(t)$ approaches a steady state value, which is denoted here by

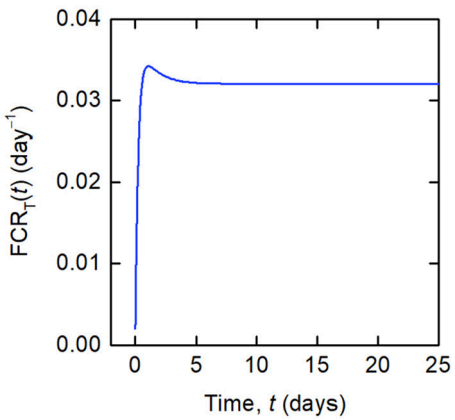

FIGURE 4 | Simulation of $\text{FCR}_{\text{T}}(t)$ given by Equations (12) and (8), for the parameter values in **Table 1** and dose $D = 0.01$ μmol.

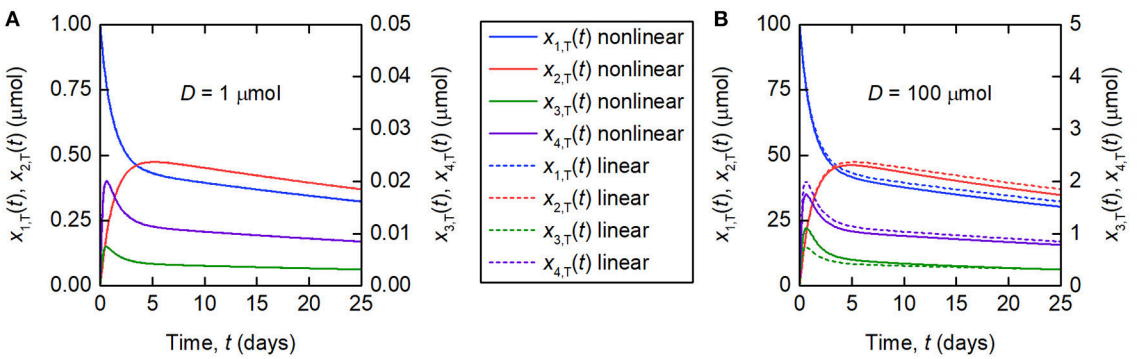

FIGURE 3 | Simulations of the quantities of tracer in each compartment after administration at $t = 0$ days, for a tracer dose of **(A)** 1 μmol and **(B)** 100 μmol. The nonlinear model (Equations 3–4) is represented by solid lines and the linearized model (Equations 6) by dashed lines. The linearized model is valid for the smaller dose but not for the larger dose. Note the different scales for $x_{1,\text{T}}(t)$ and $x_{2,\text{T}}(t)$, and $x_{3,\text{T}}(t)$ and $x_{4,\text{T}}(t)$, respectively.

$FCR_{T,\infty}$:

$$FCR_{T,\infty} = \lim_{t\to\infty} \frac{k_{03}x_{3,T}(t)}{x_{1,T}(t)}. \quad (9)$$

Solving Equations (6) gives

$$x_{i,T}(t) = A_{i1}\exp(\lambda_1 t) + A_{i2}\exp(\lambda_2 t) + A_{i3}\exp(\lambda_3 t) + A_{i4}\exp(\lambda_4 t), i = 1,\ldots,4, \quad (10)$$

where A_{ij} and λ_j ($j = 1,\ldots,4$) are expressions in terms of the model parameters and $|\lambda_1| > |\lambda_2| > |\lambda_3| > |\lambda_4|$. After sufficient time, $x_{i,T}(t)$ can be approximated by $A_{i4}\exp(\lambda_4 t)$; thus, $FCR_{T,\infty}$ is given by

$$FCR_{T,\infty} = k_{03}\frac{A_{34}\exp(\lambda_4 t)}{A_{14}\exp(\lambda_4 t)} = k_{03}\frac{A_{34}}{A_{14}}. \quad (11)$$

The expressions for A_{34} and A_{14} in terms of the model parameters are extremely long. The Mathematica (27) code for generating the expressions for A_{ij} and λ_j is provided in the **Supplementary Material**.

Noting that there is only elimination from the system and no input for $t > 0$, $FCR_T(t)$ is equal to the rate of change of radiolabeled IgG in all compartments, divided by the quantity of radiolabeled IgG in plasma:

$$FCR_T(t) = \frac{-\left(\dot{x}_{1,T}(t) + \dot{x}_{2,T}(t) + \dot{x}_{3,T}(t) + \dot{x}_{4,T}(t)\right)}{x_{1,T}(t)} = \frac{-\dot{y}_2(t)}{y_1(t)}. \quad (12)$$

From Equation (12), it can be seen that $FCR_T(t)$ is equal to the slope of the observation $y_2(t)$ divided by $y_1(t)$, showing how $FCR_T(t)$ can be obtained from the observations $y_1(t)$ and $y_2(t)$. In practice, the experimenter obtains a value for $FCR_T(t_N)$, where t_N is a time toward the end of the experiment, such that $FCR_T(t_N)$ can be assumed a close approximation of $FCR_{T,\infty}$. Henceforth, the quantity obtained from experiments, $FCR_T(t_N)$, is referred to simply as FCR_T.

It is also possible to derive an expression for the FCR with respect to the endogenous IgG, FCR_E. If the endogenous IgG is assumed to remain in steady state, then from the definition of the FCR,

$$FCR_E = \frac{k_{03}\hat{x}_3}{\hat{x}_1}, \quad (13)$$

where $\hat{x}_1$ and $\hat{x}_3$ are the quantities of IgG in compartments 1 and 3 in steady state, given by Equations (2). Substituting the expression for $\hat{x}_3$ from Equations (2) into Equation (13), eliminating I_0 in favor of $\hat{x}_1$ using the first equation of Equations (2), and setting $\hat{x}_1 = x_{1,E}$, gives the following expression for the FCR_E in terms of the quantity of IgG in plasma, $x_{1,E}$:

$$\begin{aligned} FCR_E = \frac{1}{2k_{on}x_{1,E}}\Big(&k_{31}k_{on}x_{1,E} - k_{14}k_{on}R_{tot} - k_{03}k_{14}v_3 \\ &- k_{03}k_{off}v_3 + \Big\{4k_{03}k_{31}(k_{14}+k_{off})k_{on}x_{1,E}v_3 \\ &+ (-k_{31}k_{on}x_{1,E} + k_{14}k_{on}R_{tot} + k_{03}k_{14}v_3 \\ &+ k_{03}k_{off}v_3)^2\Big\}^{1/2}\Big). \end{aligned} \quad (14)$$

3. RESULTS

3.1. Parameter Identification Using Tracer Timecourse Data

In this section we investigate whether it is possible to estimate unknown model parameter values by fitting the linear approximation described in section 2.4 to the timecourse data described in section 2.2. Firstly, a structural identifiability analysis is performed. Parameter values are then estimated from the data by fitting the linearized model described by Equations (6) to the data.

3.1.1. Structural Identifiability Analysis

Structural identifiability addresses the question of whether model parameters can be uniquely identified from the available observations, under the assumption of the availability of ideal (i.e. noise-free) and continuous observational data. Here we determine which of the model parameters are structurally uniquely identifiable from the observations $y_1(t)$ and $y_2(t)$, given by Equations (4–6). The unknown parameter vector is given by $\boldsymbol{\theta} = (k_{21}, k_{31}, k_{12}, k_{14}, k_{03}, k_{43}, k_{34})^T$.

The transfer function method is used (28). To apply this approach the system described by Equations (4–6) is re-written in vector-matrix notation as

$$\begin{aligned} \dot{\boldsymbol{x}}_T(t,\boldsymbol{\theta}) &= \boldsymbol{A}(\boldsymbol{\theta})\boldsymbol{x}_T(t) + \boldsymbol{B}(\boldsymbol{\theta})u(t) \\ \boldsymbol{x}_T(0,\boldsymbol{\theta}) &= 0 \\ \boldsymbol{y}(t,\boldsymbol{\theta}) &= \boldsymbol{C}(\boldsymbol{\theta})\boldsymbol{x}_T(t), \end{aligned} \quad (15)$$

where $\boldsymbol{x}_T(t,\boldsymbol{\theta}) = \left(x_{1,T}(t), x_{2,T}(t), x_{3,T}(t), x_{4,T}(t)\right)^T$ and $\boldsymbol{y}(t,\boldsymbol{\theta}) = \left(y_1(t), y_2(t)\right)^T$ are column vectors representing the state vector and the observation vector, respectively, and $u(t)$ represents the single input to the system, an impulse at time $t = 0$, given by $u(t) = \delta(t)$. $\boldsymbol{A}(\boldsymbol{\theta})$ is a 4×4 matrix, $\boldsymbol{B}(\boldsymbol{\theta})$ is a column vector and $\boldsymbol{C}(\boldsymbol{\theta})$ is a 2×4 matrix. $\boldsymbol{A}(\boldsymbol{\theta})$, $\boldsymbol{B}(\boldsymbol{\theta})$, and $\boldsymbol{C}(\boldsymbol{\theta})$ are given by

$$\begin{aligned} \boldsymbol{A}(\boldsymbol{\theta}) &= \begin{pmatrix} -\left(k_{21}+k_{31}\right) & k_{12} & 0 & k_{14} \\ k_{21} & -k_{12} & 0 & 0 \\ k_{31} & 0 & -\left(k_{03}+k_{43}\right) & k_{34} \\ 0 & 0 & k_{43} & -\left(k_{14}+k_{34}\right) \end{pmatrix}, \\ \boldsymbol{B}(\boldsymbol{\theta}) &= \begin{pmatrix} D \\ 0 \\ 0 \\ 0 \end{pmatrix}, \\ \boldsymbol{C}(\boldsymbol{\theta}) &= \begin{pmatrix} \frac{1}{D} & 0 & 0 & 0 \\ \frac{1}{D} & \frac{1}{D} & \frac{1}{D} & \frac{1}{D} \end{pmatrix}. \end{aligned} \quad (16)$$

Note that the administration of a bolus dose of size D is now represented as an impulse at time $t = 0$, rather than a non-zero initial condition, such that $\boldsymbol{x}_T(0,\boldsymbol{\theta}) = (0,0,0,0)^T$.

Taking Laplace transforms of Equations (15), the input-output relation is given by $\boldsymbol{Y}(s) = \boldsymbol{G}(s)U(s)$, where $\boldsymbol{G}(s)$ is the transfer function matrix, given by $\boldsymbol{G}(s) = \boldsymbol{C}(\boldsymbol{\theta})(s\boldsymbol{I} - \boldsymbol{A}(\boldsymbol{\theta}))^{-1}\boldsymbol{B}(\boldsymbol{\theta})$, where $\boldsymbol{I}$ is the 4×4 identity matrix. $\boldsymbol{G}(s)$ has two elements, corresponding

to the two observed outputs, which are given by

$$G_1(s) = \frac{\phi_1 + \phi_2 s + \phi_3 s^2 + s^3}{\phi_4 + \phi_5 s + \phi_6 s^2 + \phi_7 s^3 + s^4}$$
$$G_2(s) = \frac{\phi_8 + \phi_9 s + \phi_{10} s^2 + s^3}{\phi_{11} + \phi_{12} s + \phi_{13} s^2 + \phi_{14} s^3 + s^4}, \quad (17)$$

where the coefficients of s, $\mathbf{\Phi}(\boldsymbol{\theta}) = (\phi_1(\boldsymbol{\theta}), \phi_2(\boldsymbol{\theta}), ..., \phi_{14}(\boldsymbol{\theta}))^{\mathrm{T}}$, are nonlinear expressions in the parameters. The coefficients of s, $\mathbf{\Phi}(\boldsymbol{\theta})$, are given by

$$\begin{aligned}
&\phi_1(\boldsymbol{\theta}) = k_{12}\left(k_{03}\left(k_{14}+k_{34}\right)+k_{14}k_{43}\right)\\
&\phi_2(\boldsymbol{\theta}) = k_{03}\left(k_{12}+k_{14}+k_{34}\right)+k_{14}k_{43}+k_{12}\left(k_{14}+k_{34}+k_{43}\right)\\
&\phi_3(\boldsymbol{\theta}) = k_{03}+k_{12}+k_{14}+k_{34}+k_{43}\\
&\phi_4(\boldsymbol{\theta}) = \phi_{11}(\boldsymbol{\theta}) = k_{03}k_{12}k_{31}(k_{14}+k_{34})\\
&\phi_5(\boldsymbol{\theta}) = \phi_{12}(\boldsymbol{\theta}) = k_{03}((k_{21}+k_{31})(k_{14}+k_{34})+k_{12}(k_{14}+k_{31}\\
&\quad +k_{34}))+k_{14}k_{21}k_{43}+k_{12}(k_{14}(k_{31}+k_{43})+k_{31}(k_{34}+k_{43}))\\
&\phi_6(\boldsymbol{\theta}) = \phi_{13}(\boldsymbol{\theta}) = k_{14}k_{21}+k_{14}k_{31}+k_{21}k_{34}+k_{31}k_{34}+k_{03}(k_{12}\\
&\quad +k_{14}+k_{21}+k_{31}+k_{34})+k_{14}k_{43}+k_{21}k_{43}+k_{31}k_{43}+k_{12}\\
&(k_{14}+k_{31}+k_{34}+k_{43})\\
&\phi_7(\boldsymbol{\theta}) = \phi_{10}(\boldsymbol{\theta}) = \phi_{14}(\boldsymbol{\theta}) = k_{03}+k_{12}+k_{14}+k_{21}+k_{31}\\
&\quad +k_{34}+k_{43}\\
&\phi_8(\boldsymbol{\theta}) = k_{03}(k_{12}+k_{21})(k_{14}+k_{34})+k_{14}k_{21}k_{43}\\
&\quad +k_{12}(k_{14}(k_{31}+k_{43})+k_{31}(k_{34}+k_{43}))\\
&\phi_9(\boldsymbol{\theta}) = k_{14}k_{21}+k_{14}k_{31}+k_{21}k_{34}+k_{31}k_{34}+k_{03}(k_{12}+k_{14}\\
&\quad +k_{21}+k_{34})+k_{14}k_{43}+k_{21}k_{43}+k_{31}k_{43}\\
&\quad +k_{12}(k_{14}+k_{31}+k_{34}+k_{43}).
\end{aligned} \quad (18)$$

The coefficients $\mathbf{\Phi}(\boldsymbol{\theta})$ are unique with respect to the input-output relationship represented by the transfer function. Introducing an alternative parameter vector, $\bar{\boldsymbol{\theta}} = (\bar{k}_{21}, \bar{k}_{31}, \bar{k}_{12}, \bar{k}_{14}, \bar{k}_{03}, \bar{k}_{43}, \bar{k}_{34})^{\mathrm{T}}$, and equating $\mathbf{\Phi}(\boldsymbol{\theta}) = \mathbf{\Phi}(\bar{\boldsymbol{\theta}})$, the resulting set of simultaneous equations is solved for $\boldsymbol{\theta}$ using the Solve function in Mathematica (27). The only solution is $\boldsymbol{\theta} = \bar{\boldsymbol{\theta}}$; therefore all of the parameters in $\boldsymbol{\theta}$ are structurally uniquely identifiable.

3.1.2. Parameter Estimation

The parameter vector $\boldsymbol{\theta} = (k_{21}, k_{31}, k_{12}, k_{14}, k_{03}, k_{43}, k_{34})^{\mathrm{T}}$ was estimated for each subject using unweighted least squares, by fitting the timecourse data described in section 2.2. The "true" parameter vector for an individual is denoted by $\boldsymbol{\theta}_0$. For an individual subject it is assumed that $y_i(t, \boldsymbol{\theta_0}), i = 1, 2$, is observed with error at measurement times $t_1^{(i)}, \ldots, t_{N_i}^{(i)}, i = 1, 2$, where $t_1^{(1)} = t_1^{(2)} = 0$. The observed (with error) values of $y_i(t, \boldsymbol{\theta_0}), i = 1, 2$, are now denoted by $\tilde{y}_i(t_j^{(i)}, \boldsymbol{\theta_0})$ for $i = 1, 2$ and $j = 1, \ldots, N_i$. Both outputs y_1 and y_2 were fitted simultaneously, therefore the cost functional for $\boldsymbol{\theta}$ is given by

$$J(\boldsymbol{\theta}_0, \boldsymbol{\theta}) = \sum_{i=1}^{2} J_i(\boldsymbol{\theta}_0, \boldsymbol{\theta}), \quad (19)$$

TABLE 2 | Settings for differential evolution.

	Subject					
	A	**B**	**C**	**D**	**E**	**F**
Scaling factor (*SF*)	0.5	0.5	0.7	0.5	0.5	0.7
Crossover probability (*CR*)	0.9	0.9	0.95	0.9	0.9	0.95

where

$$J_i(\boldsymbol{\theta}_0, \boldsymbol{\theta}) = \sum_{j=1}^{N_i} \left(\tilde{y}_i(t_j^{(i)}, \boldsymbol{\theta_0}) - y_i(t_j^{(i)}, \boldsymbol{\theta})\right)^2. \quad (20)$$

Differential evolution was implemented using the NonlinearModelFit function in Mathematica (27). The differential evolution algorithm was chosen because there is little information available about the parameters, in particular the parameters k_{14}, k_{03}, k_{43}, and k_{34}. Differential evolution is a stochastic, global minimization algorithm that does not require the user to specify initial guesses for the parameter values (29). All parameters were constrained to be positive. The maximum number of iterations was set to 5,000, which was sufficient for the algorithm to converge in all cases. In differential evolution an initial population of parameter vectors is generated randomly. The algorithm was run for each subject's data with integer seeds for the pseudorandom number generator between 1 and 10; thus 10 estimates for $\boldsymbol{\theta}$ were obtained for each subject.

Differential evolution maintains a population of parameter vectors which evolves iteratively. For each new generation of the algorithm, a mutant and trial vector are produced from the current generation and the trial vector is compared with a target vector from the current generation. Either the target or trial vector is selected to move forward to the new generation based on which has the smallest value of the cost function to be minimized. The scaling factor (*SF*) is used to produce the mutant vector and generally a larger value of *SF* means a broader search of the parameter space. The crossover probability (*CR*) is the probability that each element of the mutant vector is used to produce the trial vector, rather than the corresponding element of the target vector. *SF* and *CR* were tuned by trial and error for each subject. The settings $F = 0.5$ and $CR = 0.9$ were tried initially, as recommended by Storn and Price (29) for faster convergence. For subjects C and F the settings were adjusted to $F = 0.7$, for a broader search of the parameter space, and $CR = 0.95$, to speed convergence. The settings for the differential evolution algorithm are given in **Table 2**.

Each run of the algorithm, with a unique seed for the pseudorandom number generator, can produce unique parameter estimates; it is therefore recommended to perform multiple runs with unique, randomly chosen starting populations of parameter vectors (29). The parameter estimates and root mean square error (RMSE) for each run and each subject are tabulated in **Table 3**. The parameter estimates from multiple runs should be close to one another so that they can be averaged (29, 30); however, in some cases, the different runs give very different parameter estimates, implying that the algorithm has

TABLE 3 | Parameter values estimated from timecourse data.

	Run	Parameter (all have units day^{-1})							RMSE
		k_{21}	k_{31}	k_{12}	k_{14}	k_{03}	k_{43}	k_{34}	
Subject A	1	0.391	0.158	1.29	0.0628	0.261	1.88	0.206	0.0124
	2	0.391	0.159	1.29	0.0623	0.294	2.23	0.216	0.0124
	3	0.390	0.159	1.29	0.0616	0.341	2.70	0.225	0.0124
	4	0.390	0.159	1.29	0.0612	0.363	2.91	0.227	0.0124
	5	0.388	0.139	1.11	0.0699	0.0881	0.209	0.0279	0.0123
	6	0.391	0.160	1.30	0.0611	0.395	3.25	0.233	0.0124
	7	0.392	0.160	1.30	0.0616	0.365	2.95	0.229	0.0124
	8	0.386	0.159	1.27	0.0615	0.327	2.54	0.221	0.0124
	9	0.391	0.159	1.29	0.0617	0.336	2.64	0.224	0.0124
	10	0.390	0.159	1.29	0.0619	0.307	2.35	0.218	0.0124
Subject B	1	1.72	0.174	2.96	0.151	1.04	1.23	$1.09 \cdot 10^{-16}$	0.00858
	2	0.101	0.732	0.147	3.18	0.208	1.72	0.00	0.00859
	3	0.0986	1.09	0.146	2.49	0.408	20.2	7.16	0.00865
	4	1.73	0.174	2.98	0.151	1.04	1.23	0.00	0.00858
	5	1.72	0.174	2.96	0.151	1.04	1.23	0.00	0.00858
	6	1.72	0.174	2.96	0.151	1.04	1.23	0.00	0.00858
	7	1.72	0.174	2.96	0.151	1.04	1.23	0.00	0.00858
	8	1.72	0.174	2.96	0.151	1.04	1.23	$3.69 \cdot 10^{-15}$	0.00858
	9	0.101	0.732	0.147	3.18	0.208	1.72	0.00	0.00859
	10	1.72	0.174	2.96	0.151	1.04	1.23	0.00	0.00858
Subject C	1	0.0217	0.438	$6.47 \cdot 10^{-16}$	0.527	0.580	2.10	0.332	0.00682
	2	0.346	0.160	0.537	0.00	1.3126	0.447	0.0880	0.00553
	3	0.0217	0.438	$9.75 \cdot 10^{-15}$	0.527	0.580	2.10	0.332	0.00682
	4	1100	0.349	8590	0.2537	0.765	1.72	0.141	0.00780
	5	0.346	0.160	0.537	$2.81 \cdot 10^{-16}$	1.31	0.447	0.0880	0.00553
	6	203	0.349	1580	0.254	0.764	1.72	0.141	0.00780
	7	0.0217	0.438	$1.51 \cdot 10^{-16}$	0.527	0.580	2.10	0.332	0.00682
	8	0.0217	0.438	$4.65 \cdot 10^{-15}$	0.527	0.580	2.10	0.332	0.00682
	9	284	0.349	2210	0.254	0.765	1.73	0.141	0.00780
	10	0.0217	0.438	$2.75 \cdot 10^{-15}$	0.527	0.580	2.10	0.332	0.00682
Subject D	1	0.346	0.154	0.432	$2.73 \cdot 10^{7}$	20.3	80.2	0.0550	0.0136
	2	0.346	1.50	0.432	$1.45 \cdot 10^{9}$	15.3	725	68.7	0.0136
	3	0.346	0.159	0.432	$4.85 \cdot 10^{16}$	9.08	37.3	94600	0.0137
	4	0.346	0.173	0.432	$1.99 \cdot 10^{7}$	11.5	52.4	1180	0.0137
	5	0.346	0.102	0.432	$2.12 \cdot 10^{17}$	22.2	50.7	0.00	0.0136
	6	0.344	1.95	0.433	$1.16 \cdot 10^{7}$	3.90	240	208	0.0136
	7	0.346	0.0999	0.432	$1.22 \cdot 10^{6}$	15.4	34.1	3.07	0.0137
	8	0.346	0.951	0.432	$2.16 \cdot 10^{11}$	12.8	379	1710	0.0136
	9	0.134	0.242	0.429	0.435	5.49	37.1	0.00	0.0137
	10	0.347	0.142	0.432	$4.14 \cdot 10^{8}$	163	581	0.00	0.0136
Subject E	1	0.412	0.117	0.361	0.273	0.995	1.02	0.326	0.00550
	2	$1.40 \cdot 10^{-6}$	0.445	142	0.452	0.148	0.693	0.00603	0.00379
	3	$2.20 \cdot 10^{-13}$	0.445	8.71	0.452	0.148	0.692	0.00601	0.00379
	4	0.454	0.0795	0.362	$7.12 \cdot 10^{-8}$	4.51	5.30	1.12	0.00550
	5	0.454	0.0795	0.362	$2.97 \cdot 10^{-12}$	5.40	6.76	1.14	0.00550
	6	0.454	0.0795	0.362	0.0000227	3.41	3.52	1.04	0.00550

(Continued)

TABLE 3 | Continued

	Run	Parameter (all have units day^{-1})							RMSE
		k_{21}	k_{31}	k_{12}	k_{14}	k_{03}	k_{43}	k_{34}	
	7	0.00	0.454	$6.91 \cdot 10^{7}$	0.419	0.175	0.948	0.0586	0.00402
	8	0.454	0.0795	0.362	0.00	4.11	4.66	1.09	0.00550
	9	0.454	0.0795	0.362	0.0000416	3.21	3.20	1.02	0.00550
	10	0.454	0.0795	0.362	$5.41 \cdot 10^{-7}$	51.2	84.0	1.30	0.00550
Subject F	1	0.456	4.22	0.372	19.3	0.956	$1.45 \cdot 10^{7}$	$5.37 \cdot 10^{6}$	0.00686
	2	$1.23 \cdot 10^{10}$	0.532	$4.29 \cdot 10^{10}$	0.360	1690	15300	0.214	0.00286
	3	0.456	4.21	0.372	14.6	6.23	$1.21 \cdot 10^{8}$	$5.22 \cdot 10^{6}$	0.00686
	4	0.456	4.21	0.372	17.1	1.47	$1.70 \cdot 10^{7}$	$3.64 \cdot 10^{6}$	0.00686
	5	0.456	4.21	0.372	15.4	2.87	$1.40 \cdot 10^{8}$	$1.39 \cdot 10^{7}$	0.00686
	6	0.456	4.21	0.372	17.6	1.28	$1.54 \cdot 10^{7}$	$3.88 \cdot 10^{6}$	0.00686
	7	0.456	4.16	0.372	48.0	0.364	185	407	0.00687
	8	0.456	4.22	0.372	16.1	2.02	$1.42 \cdot 10^{7}$	$1.42 \cdot 10^{7}$	0.00686
	9	$4.97 \cdot 10^{8}$	0.531	$1.73 \cdot 10^{9}$	0.360	33600	304000	0.214	0.00286
	10	0.456	4.21	0.372	15.7	2.51	$1.44 \cdot 10^{8}$	$1.65 \cdot 10^{7}$	0.00686

difficulty finding the global minimum and that there may be many local minima. It is therefore not certain that the global minimum has been found for each subject. It is also possible that certain parameters are highly correlated, such that different parameter vectors produce very similar model outputs. This is reflected in the diversity of parameter vectors obtained within subjects using differential evolution.

In some cases the model parameters are estimated to be zero, or very close to zero, for example k_{34} for subject B, k_{12} and k_{14} for subject C, k_{34} for subject D, and k_{21} and k_{14} for subject E. For each of these subjects the data can be well represented by a reduced model in which either IgG-FcRn binding is irreversible ($k_{34} = 0$), there is no transfer from the peripheral compartment to plasma ($k_{12} = 0$) or vice versa ($k_{21} = 0$), or bound IgG molecules are not recycled into plasma ($k_{14} = 0$). This result suggests that the model complexity is not supported by the available data.

The data and the model outputs using the parameter estimates in **Table 3** are plotted in **Figure 5**. In each panel of **Figure 5**, the model outputs $y_1(t)$ and $y_2(t)$ are plotted for each of the estimated parameter vectors from 10 runs. The model outputs are very similar for all of the estimated parameter vectors for an individual. For some subjects there are small but noticeable differences between the fits, for example: in the first and last 5 days of $y_2(t)$ for subject A; in the first 2 days of $y_1(t)$ for subject B; for all of $y_1(t)$ and the latter part of $y_2(t)$ for subject C; between days 2 and 6 for $y_1(t)$ and the initial 2 days of $y_2(t)$ for subject E; and the first 10 days and final 5 days of $y_2(t)$ for subject F. The similarity between the outputs for the parameter estimates obtained across different runs is shown by the similar values of RMSE within each subject. The model appears to fit the data reasonably well and in some subjects extremely well.

The results of the multiple runs of differential evolution show that in many cases, highly different parameter vectors produce very similar model outputs. The spread of the parameter estimates from multiple runs is conveyed using the coefficient of variation (CV), that is, the standard deviation of the estimates of a parameter from 10 runs, divided by the mean of those estimates. The CV is tabulated in **Table 4**. For some parameters and subjects, the estimates for the parameters have a small CV, for example the first four parameters for subject A and parameter k_{12} for subject D. In other instances however the CV is much larger, reflecting the highly different estimates obtained for these parameters. The similarly high quality fits produced by diverse parameter vectors implies that, whilst the parameters are structurally identifiable, they are not all *practically* identifiable for the quality of data that are available.

3.2. Parameter Identification Using Fractional Catabolic Rate Data

Authors who have studied a two-compartment model of IgG metabolism have previously estimated parameters from FCR vs. plasma IgG concentration data (16, 21). In this section we investigate whether it is possible to estimate parameters of the four-compartment model from these data, which are described in section 2.2. In section 2.6 two expressions for the FCR were introduced: the FCR of the tracer (Equation 11) and the FCR of the endogenous IgG in steady state (Equation 14). In practice FCR_T is measured; however it is difficult to obtain a closed form expression for FCR_T. In contrast, we can easily obtain an expression for FCR_E in terms of the model parameters and the quantity of endogenous IgG in plasma, $x_{1,E}$, as given by Equation (14). In this section model parameters are estimated by fitting the expression for FCR_E vs. $x_{1,E}$ Equation (14) to the FCR_T vs. $x_{1,E}$ data. It is assumed that FCR_E is a good approximation to FCR_T and the parameter estimates are validated in section 3.2.3 using synthetic data.

3.2.1. Structural Identifiability Analysis

The relationship between FCR_E and $x_{1,E}$ is given by Equation (14). Given that the parameters k_{on} and v_3 only appear in the model (Equations 3) as the ratio k_{on}/v_3, we re-write Equation (14), defining $\phi_1 = k_{on}/v_3$, giving

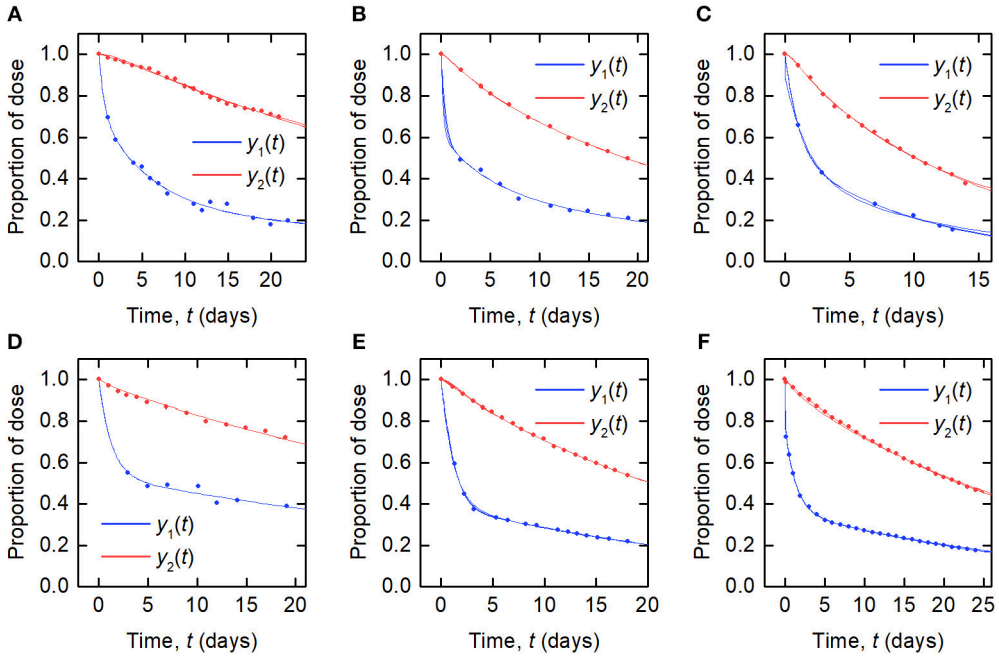

FIGURE 5 | Timecourse data [$y_1(t)$ blue circles; $y_2(t)$ red circles] and model fits [$y_1(t)$ blue line; $y_2(t)$ red line] for **(A–F)** subjects A–F.

$$\mathrm{FCR_E} = \frac{1}{2\phi_1 x_{1,\mathrm{E}}}\Bigg(k_{31}\phi_1 x_{1,\mathrm{E}} - k_{14}\phi_1 R_{\mathrm{tot}} - k_{03}k_{14} - k_{03}k_{\mathrm{off}} + \sqrt{4k_{03}k_{31}\left(k_{14}+k_{\mathrm{off}}\right)\phi_1 x_{1,\mathrm{E}} + \left(-k_{31}\phi_1 x_{1,\mathrm{E}} + k_{14}\phi_1 R_{\mathrm{tot}} + k_{03}\left(k_{14}+k_{\mathrm{off}}\right)\right)^2}\Bigg). \tag{21}$$

We wish to know whether the parameter vector $\boldsymbol{\phi} = \left(\phi_1, k_{31}, k_{14}, R_{\mathrm{tot}}, k_{03}, k_{\mathrm{off}}\right)^{\mathrm{T}}$ is structurally identifiable with respect to the relationship in Equation (21). The structural identifiability problem amounts to determining whether there exists an alternative parameter vector $\bar{\boldsymbol{\phi}} = \left(\bar{\phi}_1, \bar{k}_{31}, \bar{k}_{14}, \bar{R}_{\mathrm{tot}}, \bar{k}_{03}, \bar{k}_{\mathrm{off}}\right)^{\mathrm{T}}$ such that $\mathrm{FCR_E}(x_{1,\mathrm{E}}, \boldsymbol{\phi}) = \mathrm{FCR_E}(x_{1,\mathrm{E}}, \bar{\boldsymbol{\phi}})$.

From Equations (13) and (2),

$$\mathrm{FCR_E} = \frac{I_0}{\hat{x}_1}. \tag{22}$$

I_0 is given in terms of $\hat{x}_1$ by the solution of the following quadratic equation, obtained by rearranging the first equation of Equations (2) and setting $\phi_1 = k_{\mathrm{on}}/v_3$:

$$-\phi_1 I_0^2 + \left(-k_{03}\left(k_{14}+k_{\mathrm{off}}\right) + \phi_1\left(k_{31}\hat{x}_1 - k_{14}R_{\mathrm{tot}}\right)\right) I_0 + k_{03}k_{31}\left(k_{14}+k_{\mathrm{off}}\right)\hat{x}_1 = 0. \tag{23}$$

Substituting $\mathrm{FCR_E}\hat{x}_1$ in place of I_0 and setting $\hat{x}_1 = x_{1,\mathrm{E}}$ gives the following quadratic equation in $\mathrm{FCR_E}$:

$$-\phi_1 x_{1,\mathrm{E}}^2 \mathrm{FCR_E^2} + \left(-k_{03}\left(k_{14}+k_{\mathrm{off}}\right) + \phi_1\left(k_{31}x_{1,\mathrm{E}} - k_{14}R_{\mathrm{tot}}\right)\right) x_{1,\mathrm{E}}\mathrm{FCR_E} + k_{03}k_{31}\left(k_{14}+k_{\mathrm{off}}\right)x_{1,\mathrm{E}} = 0. \tag{24}$$

Dividing Equation (24) throughout by the coefficient of $\mathrm{FCR_E^2}$ gives

$$\mathrm{FCR_E^2} + \left(\frac{k_{03}\left(k_{14}+k_{\mathrm{off}}\right) - k_{31}\phi_1 x_{1,\mathrm{E}} + k_{14}\phi_1 R_{\mathrm{tot}}}{\phi_1 x_{1,\mathrm{E}}}\right)\mathrm{FCR_E} - \frac{k_{03}k_{31}\left(k_{14}+k_{\mathrm{off}}\right)}{\phi_1 x_{1,\mathrm{E}}} = 0. \tag{25}$$

The expression for $\mathrm{FCR_E}$ given by Equation (21) is one of the two solutions of Equation (25). We therefore wish to know whether there exists an alternative parameter vector $\bar{\boldsymbol{\phi}}$ such that,

$$\mathrm{FCR_E^2} + \left(\frac{k_{03}\left(k_{14}+k_{\mathrm{off}}\right) - k_{31}\phi_1 x_{1,\mathrm{E}} + k_{14}\phi_1 R_{\mathrm{tot}}}{\phi_1 x_{1,\mathrm{E}}}\right)\mathrm{FCR_E} - \frac{k_{03}k_{31}\left(k_{14}+k_{\mathrm{off}}\right)}{\phi_1 x_{1,\mathrm{E}}} = \mathrm{FCR_E^2} + \left(\frac{\bar{k}_{03}\left(\bar{k}_{14}+\bar{k}_{\mathrm{off}}\right) - \bar{k}_{31}\bar{\phi}_1 x_{1,\mathrm{E}} + \bar{k}_{14}\bar{\phi}_1\bar{R}_{\mathrm{tot}}}{\bar{\phi}_1 x_{1,\mathrm{E}}}\right)\mathrm{FCR_E} - \frac{\bar{k}_{03}\bar{k}_{31}\left(\bar{k}_{14}+\bar{k}_{\mathrm{off}}\right)}{\bar{\phi}_1 x_{1,\mathrm{E}}}. \tag{26}$$

TABLE 4 | Coefficients of variation of parameter estimates obtained from 10 runs of differential evolution, for each of subjects A–F.

Parameter	Coefficient of variation					
	A	B	C	D	E	F
k_{21}	0.00404	0.634	2.18	0.206	0.691	3.03
k_{31}	0.0405	0.905	0.312	1.24	0.907	0.446
k_{12}	0.0458	0.642	2.18	0.00277	3.16	3.03
k_{14}	0.0417	1.38	0.643	2.58	1.33	0.792
k_{03}	0.280	0.461	0.373	1.71	2.12	3.00
k_{43}	0.360	1.85	0.399	1.15	2.32	1.14
k_{34}	0.305	3.16	0.502	3.05	0.758	1.01

From the uniqueness of interpolating polynomials (31, p. 98), the coefficients of the quadratic in Equation (25) are unique, therefore the problem amounts to solving the simultaneous equations:

$$\begin{aligned}&\frac{k_{03}\left(k_{14}+k_{\text{off}}\right)-k_{31}\phi_1 x_{1,\text{E}}+k_{14}\phi_1 R_{\text{tot}}}{\phi_1 x_{1,\text{E}}}\\&=\frac{\bar{k}_{03}\left(\bar{k}_{14}+\bar{k}_{\text{off}}\right)-\bar{k}_{31}\bar{\phi}_1 x_{1,\text{E}}+\bar{k}_{14}\bar{\phi}_1\bar{R}_{\text{tot}}}{\bar{\phi}_1 x_{1,\text{E}}}\\&-\frac{k_{03}k_{31}\left(k_{14}+k_{\text{off}}\right)}{\phi_1 x_{1,\text{E}}}=-\frac{\bar{k}_{03}\bar{k}_{31}\left(\bar{k}_{14}+\bar{k}_{\text{off}}\right)}{\bar{\phi}_1 x_{1,\text{E}}}.\end{aligned}\tag{27}$$

The solution was found using the SolveAlways function in Mathematica. The only solution to Equations (27), for all values of $x_{1,\text{E}}$, is given by

$$\begin{aligned}\bar{k}_{31}&=k_{31}\\\bar{k}_{14}\bar{R}_{\text{tot}}&=k_{14}R_{\text{tot}}\\\frac{\bar{k}_{03}\left(\bar{k}_{14}+\bar{k}_{\text{off}}\right)}{\bar{\phi}_1}&=\frac{k_{03}\left(k_{14}+k_{\text{off}}\right)}{\phi_1}.\end{aligned}\tag{28}$$

Therefore, only k_{31} and the expressions $k_{14}R_{\text{tot}}$ and $k_{03}\left(k_{14}+k_{\text{off}}\right)/\phi_1$, containing original parameter combinations, are structurally identifiable with respect to the relationship between FCR_E and $x_{1,\text{E}}$.

3.2.2. Parameter Estimation

Having analyzed the structural identifiability of the expression for FCR_E vs. $x_{1,\text{E}}$, it becomes clear that we can rewrite the expression in Equation (14) by combining parameters into new structurally identifiable parameters, as follows:

$$\begin{aligned}\text{FCR}_\text{E}(x_{1,\text{E}},\boldsymbol{\psi})=\frac{1}{2x_{1,\text{E}}}\Big(&k_{31}x_{1,\text{E}}-\psi_1-\psi_2\\&+\sqrt{k_{31}^2x_{1,\text{E}}^2+2k_{31}x_{1,\text{E}}\left(\psi_1-\psi_2\right)+\left(\psi_1+\psi_2\right)^2}\Big),\end{aligned}\tag{29}$$

TABLE 5 | Parameter estimates from fitting FCR_E expression to FCR_T vs. $x_{1,\text{E}}$ data.

Parameter	Units	Estimate	Standard error	95% confidence interval
ψ_1	$\mu\text{mol day}^{-1}$	7.47	2.74	(1.93, 13.0)
ψ_2	$\mu\text{mol day}^{-1}$	25.7	6.656	(12.3, 39.2)
k_{31}	day^{-1}	0.154	0.00969	(0.135, 0.174)

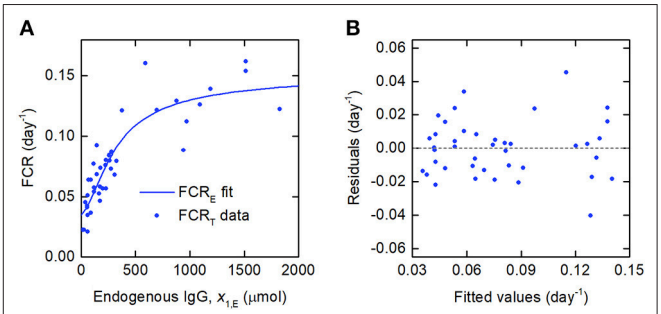

FIGURE 6 | (A) Expression for FCR_E vs. $x_{1,\text{E}}$, given by Equation (29), fitted to FCR_T vs. $x_{1,\text{E}}$ data from Waldmann and Strober (21). **(B)** Residuals vs. fitted values.

where

$$\begin{aligned}\psi_1&=\frac{k_{03}v_3\left(k_{14}+k_{\text{off}}\right)}{k_{\text{on}}}\\\psi_2&=k_{14}R_{\text{tot}}\end{aligned}\tag{30}$$

are uniquely identifiable parameters. ψ_1 and ψ_2 have units of $\mu\text{mol day}^{-1}$. The parameter vector to be estimated is now $\boldsymbol{\psi}=\left(k_{31},\psi_1,\psi_2\right)$.

It is assumed that Equation (29) is a close approximation to the relationship between the measured FCR_T and $x_{1,\text{E}}$. Waldmann and Strober (21) provide FCR_T vs. plasma IgG concentration data. The plasma concentrations of endogenous IgG were multiplied by the average plasma volume v_1, from **Table 1**, in order to obtain the quantity of endogenous IgG in plasma, $x_{1,\text{E}}$. The data for FCR_T vs. $x_{1,\text{E}}$ were then fitted using the interior point algorithm implemented within the NonlinearModelFit function in Mathematica. The starting value for the minimization was set to 1 for each parameter. The parameter estimates were constrained to be positive.

Since the data were obtained from 41 individuals, the estimated parameter values are assumed to represent the average parameter values within the population. The parameter estimates and their standard errors are provided in **Table 5**. The fitted expression given by Equation (29) is plotted alongside the data in **Figure 6A**. The residuals vs. the fitted values are plotted in **Figure 6B**. On inspection, the model appears to fit the data well. The residuals appear reasonably homoscedastic and there is no obvious autocorrelation.

3.2.3. Validation of Parameter Estimates

There are several issues that may cause the estimates of k_{31}, ψ_1, and ψ_2 to be inaccurate. Firstly, the data were obtained from a

sample of 41 individuals, each with their own unique parameter vector; this variability is not accounted for by the estimation procedure. Secondly, the parameters were estimated by fitting the expression for FCR_E vs. $x_{1,E}$; however the data are for the FCR_T, which is not equivalent to the FCR_E. In addition, the FCR_T is in practice calculated from measurements of radioactivity in plasma and urine; the form of the measurement errors is therefore not clear.

Due to the aforementioned issues, the validity of the parameter estimates obtained in section 3.2.2 was investigated by estimating the parameters from synthetic data. It is assumed that the parameter values in **Table 5** are true population parameter values. Data for FCR_T vs. $x_{1,E}$ were simulated according to the experimental methodology, described by Waldmann and Strober (21). The data were simulated for 100 sets of 41 subjects. The parameter values were then estimated from the synthetic data, generating 100 estimates for $\boldsymbol{\psi} = (k_{31}, \psi_1, \psi_2)$.

In order to simulate the FCR_T data, parameter values are required for all model parameters (see Equations 1), not just k_{31}, ψ_1, and ψ_2. Population parameter values are therefore required for all model parameters in order to randomly generate unique parameter vectors for individual subjects. The population parameter values for k_{21}, k_{12}, k_{14}, k_{03}, and k_{off} were fixed to the values from the literature in **Table 1**. The population value of k_{31} was fixed to the estimated value in **Table 5**. The population values of R_{tot} and k_{on}/v_3 were calculated by substituting the previously fixed parameter values into Equations (30) and solving. In this way, a population parameter vector was found, for which k_{31}, ψ_1, and ψ_2 are equal to their estimated values. Unique parameter values for 41 individuals were randomly generated from a lognormal distribution, with the median given by the population parameter values. The variance was tuned by trial and error in order to replicate the size of the errors seen in the real data. This process was repeated to produce 100 sets of 41 individual parameter vectors and thus 100 sets of FCR_T vs. $x_{1,E}$ data. Full details of how the synthetic data were generated are provided in the Mathematica code in the **Supplementary Material**.

The parameter estimates as a proportion of the true parameter values are plotted in **Figure 7**, showing the spread of the parameter estimates. It is clear from this plot that the parameter k_{31} is estimated with higher precision than ψ_1 and ψ_2. The sample mean (μ), sample standard deviation (s.d.), bias (b), and variability (v) of the parameter estimates are given in **Table 6**. The bias is given by

$$b = \mu - p, \tag{31}$$

where p is the true value of the parameter. The variability is given by

$$v = \frac{\sqrt{\text{s.d.}^2 + b^2}}{p}. \tag{32}$$

Variability as defined by Equation (32) has been used by Chen et al. (32) to evaluate the performance of estimation methods when the assumptions relied upon by the methods, in particular relating to noise, are violated. A larger value of v represents a worse performance of an estimation method. The results suggest

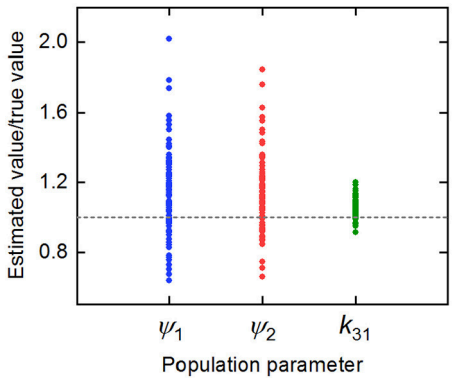

FIGURE 7 | Parameter estimates for k_{31}, ψ_1, and ψ_2 divided by the true parameter value.

TABLE 6 | Mean, standard deviation, bias, and variability of the estimates of k_{31}, ψ_1, and ψ_2.

	Parameter		
	k_{31}	ψ_1	ψ_2
Mean	0.162	8.55	29.0
Standard deviation	0.00758	1.75	5.24
Bias	0.00841	1.08	3.31
Variability	0.0735	0.275	0.241

that k_{31} has been estimated with a good level of accuracy ($v = 0.0735$), but that the parameters ψ_1 and ψ_2 were estimated with a higher level of variability. Based on this result, a future study may look at improving experimental design, for example by increasing the number of subjects, in order to improve upon the variability of the estimates of ψ_1 and ψ_2.

3.3. Simulation of IgG Responses in Multiple Myeloma

It has been shown that parameter estimates obtained using timecourse data are not robust; however, the parameters k_{31}, ψ_1, and ψ_2 may be obtained with reasonably low variability using FCR data. The results from fitting the timecourse data suggest that the model (Equations 1) may be overparameterized with respect to the available data; we therefore ask whether the plasma IgG response can be sufficiently determined using only the parameters k_{21}, k_{12}, k_{31}, ψ_1, and ψ_2.

Firstly we investigate the plasma IgG response given by the full system model (Equations 1), when the parameters k_{31}, ψ_1, and ψ_2 are equal to the values estimated in section 3.2.2. Random values were generated for certain model parameters and the remaining parameter values calculated so that k_{31}, ψ_1, and ψ_2 are equal to their estimated values. Three parameters (not including both R_{tot} and k_{14}) out of k_{03}, R_{tot}, k_{off}, k_{14}, and k_{on}/v_3 were fixed to randomly generated values and substituted into Equations (30), yielding a linear system of two equations in two unknowns. Equations (30) were then solved for the remaining two parameters. There are seven sets of three parameters from

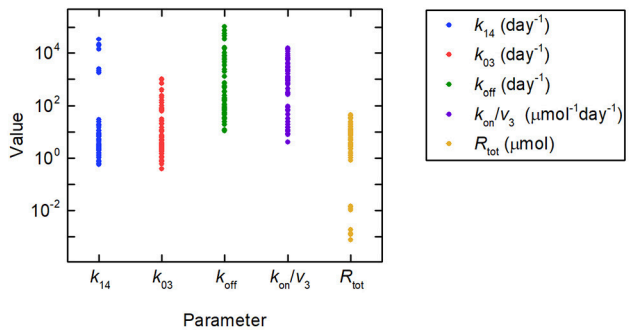

FIGURE 8 | Parameter values used to simulate IgG responses in multiple myeloma, plotted on a logarithmic scale. The method used to generate the parameter values is described in section 3.3. The model predictions generated using these parameter values are shown in **Figure 9A**. Despite the large amount of variation in the parameter values, the model predictions for plasma IgG are extremely similar.

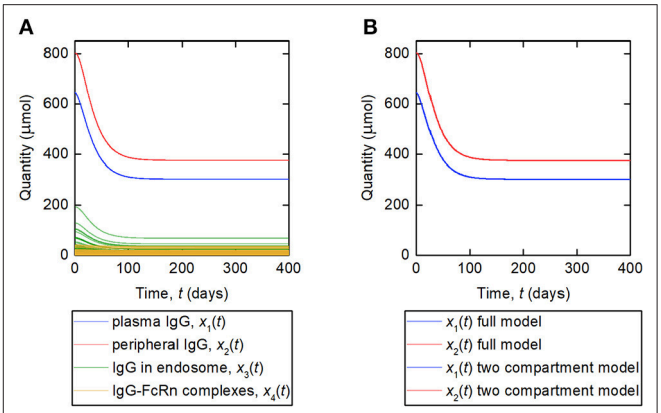

FIGURE 9 | (A) Simulation of responses of plasma IgG [$x_1(t)$], peripheral IgG [$x_2(t)$], IgG in endosomes [$x_3(t)$], and IgG bound to FcRn in endosomes [$x_4(t)$]. The scenario shown represents a decreasing tumor burden during therapy. Each variable is simulated for 70 unique parameter vectors. **(B)** Simulation of responses of plasma IgG [$x_1(t)$], peripheral IgG [$x_2(t)$], compared for the four-compartment model and the proposed two compartment model. The responses are indistinguishable by inspection for the two models.

k_{03}, R_{tot}, k_{off}, k_{14}, and k_{on}/v_3, which can be fixed to give the remaining two parameters. Parameters were generated 10 times, as described, for each of these seven sets, giving 70 parameter vectors in total. The randomly generated parameter values were obtained by assuming a lognormal distribution, in order to ensure positivity, with median set to the parameter value from the literature, given in **Table 1**, and variance 1. The values generated in this way for the parameters k_{03}, R_{tot}, k_{off}, k_{14}, and k_{on}/v_3 are depicted in **Figure 8**, showing the extremely wide range of parameter values used. The parameter k_{31} was set to the estimated value given in **Table 5**. The values for k_{21} and k_{12} were set to the values given in **Table 1**.

In order to simulate the model under realistic clinical conditions, a model for the IgG synthesis rate in multiple myeloma was used, which has been found to predict responses consistent with real patient data (33). The IgG synthesis rate is described by

$$I(t) = (I_0 - I_\infty)\exp(-k_{kill}t) + I_\infty. \tag{33}$$

The following parameter values were used to produce the simulation: $I_0 = 76$ μmol day^{-1}, $I_\infty = 26.5$ μmol day^{-1}, and $k_{kill} = 0.055$ day^{-1} (33).

A simulation of the responses in all four model compartments is shown in **Figure 9A**. Each variable is simulated for 70 unique parameter vectors. The predicted trajectories for plasma IgG and peripheral IgG, respectively, are extremely similar for all 70 parameter vectors; however there is some variation in the responses of IgG in intracellular endosomes, particularly the IgG that is not bound to FcRn receptors. The simulation suggests that, under the investigated conditions, the response in the plasma compartment is relatively insensitive to changes in the individual parameters k_{03}, R_{tot}, k_{off}, k_{14}, and k_{on}/v_3, provided that the parameters k_{31}, ψ_1, and ψ_2 are fixed. The maximal difference between any two trajectories for $x_1(t)$ at any simulated time point is 0.2%.

The lack of variation within the predicted responses for plasma and peripheral IgG, when parameters k_{21}, k_{12}, k_{31}, ψ_1, and ψ_2 are fixed, suggests that it may be possible to simulate these two variables using a reduced order model based upon the newly derived expression for the FCR (Equation 29). The equations for this model are given by

$$\begin{aligned}\dot{x}_1(t) &= -\left(k_{21} + f\left(x_1\left(t\right)\right)\right)x_1(t) + k_{12}x_2(t) + k_{14}x_4(t) + I(t)\\ \dot{x}_2(t) &= k_{21}x_1(t) - k_{12}x_2(t),\end{aligned} \tag{34}$$

where

$$f\left(x_1\left(t\right)\right) = \frac{1}{2x_1(t)}\left(k_{31}x_1(t) - \psi_1 - \psi_2 + \sqrt{k_{31}^2x_1(t)^2 + 2k_{31}x_1(t)\left(\psi_1 - \psi_2\right) + \left(\psi_1 + \psi_2\right)^2}\right). \tag{35}$$

The assumption behind this model is that the fractional rate of IgG catabolism is equal to its fractional rate of catabolism at steady state. A simulation of this model, alongside the original four-compartment model, is shown in **Figure 9B**. The model is simulated with values of k_{21} and k_{12} from **Table 1** and all other parameter values from **Table 5**. The responses for $x_1(t)$ and $x_2(t)$ are very similar for the two models and appear overlayed in **Figure 9B**. The maximal difference between $x_1(t)$ predicted by the two-compartment model and $x_1(t)$ predicted by the four-compartment model, for any of the 70 parameter vectors used and at any simulated time point, is 0.2%. The responses are indistinguishable by inspection for the two models.

The proposed two-compartment model is based upon the assumption that the fractional rate of IgG catabolism is equal to its fractional rate of catabolism in steady state. When the system is in steady state, this assumption is of course true. However, faster dynamics, caused by a rapid change in the IgG synthesis rate, will cause this assumption to progressively weaken. Further study of the proposed model is required to analyse its relationship with

the original four-compartment model and to determine under what conditions the proposed model predictions are within an acceptable region of the four-compartment model predictions.

4. DISCUSSION

The motivation behind the research presented in this paper was to investigate a model suitable for predicting IgG responses in patients with IgG multiple myeloma. When producing predictive simulations of a biomedical system, it is important to know the level of confidence in the model parameter values.

There are numerous published models of FcRn-mediated recycling of IgG in the literature, some of which are cited in the Introduction. Most of these models were developed for IgG-based therapeutic monoclonal antibodies and may not be suitable for characterizing endogenous IgG. Those models characterizing endogenous IgG, for example the models of Li et al. (34) and Chen and Balthasar (10), rely upon a mixture of animal and human data for sourcing parameter values.

For example, parameter values provided by Li et al. (34) for endogenous IgG were taken from the literature, apart from the catabolic clearance (corresponding to k_{03} of the present study), the vascular reflection coefficient (not included in our model), and the recycling rate constant (corresponding to k_{14} of the present study). These parameter values were obtained by manually varying the parameters within the model until the results showed a mean half-life of 21 days, a mean IgG synthesis rate of 34 $\mathrm{mg\,kg^{-1}\,day^{-1}}$ and a realistic fold reduction in IgG concentration when FcRn is not present. The values used for the half-life and synthesis rate are those obtained from normal human data by Waldmann and Strober (21) and Waldmann and Terry (23).

One of the problems with the approach taken in previous papers is that, whilst parameter values have been found that provide a half-life of 21 days for an IgG synthesis rate of 34 $\mathrm{mg\,kg^{-1}\,day^{-1}}$, it is not clear what would happen to the half-life when the IgG synthesis rate increases or decreases, under the obtained parameter values. This approach is therefore akin to fitting a model to a curve having only one data point. The nonlinear relationship between synthesis or concentration of IgG and its half-life, which is fundamental to the FcRn-IgG recycling system, may therefore not be captured accurately using this approach.

Another issue with this earlier approach is that it requires the parameter values obtained from the literature to be fixed while the remaining values are varied, therefore implicitly assuming complete confidence in the fixed parameter values that were sourced in the literature. One would question what would happen if one or more of these parameter values were inaccurate by, say, 10% or more, what would be the effect on the corresponding values obtained for k_{14} and k_{03}?

Having considered the models available in the literature and their issues in respect of parameter identifiability, we identified the need for a semi-mechanistic model with parameter values obtained using only *in vivo* human data. This approach necessitated a simpler model than those available in the literature and previously discussed. The model studied in this paper is therefore missing some of the mechanisms of the more complex models. However, simplified compartmental models can often be derived from complex physiologically-based models by lumping compartments and processes. Lumped models may be adequate for describing processes of interest, for example responses in a central/plasma compartment. Fronton et al. (14) demonstrate the correspondence between a physiologically-based model and several compartmental model structures for IgG. A similar study could be performed using the models presented in this paper in future work.

In this paper, two observed model outputs were considered: the timecourse of the proportion of a dose of IgG remaining in plasma and in the body of an individual subject; and the FCR vs. the quantity of endogenous IgG in plasma, measured in a cohort of subjects with a range of plasma IgG concentrations. We derived mathematical descriptions of these experimental observations based on the underlying model. Structural identifiability analysis was performed with respect to these observations in order to determine which parameters are structurally uniquely identifiable from the available outputs.

In section 3.1 we estimated parameter values using data for the timecourse of an administered dose of radiolabeled IgG in plasma and in the body. We found that all parameters of the linearized model are structurally globally identifiable. Whilst the model is capable of fitting the data well, the results of 10 runs of differential evolution suggest that the parameter estimates are not robust. Highly different parameter vectors, as illustrated by the relative standard deviations of parameter estimates from 10 runs, produce similarly excellent fits to the data. These results suggest that the available data do not support the complexity of the model. A future study may apply a systematic analysis of model sensitivity and parameter correlations, for example using the profile-likelihood method of Raue et al. (35) or generalized sensitivity functions of Thomaseth and Cobelli (36) [extended to multiple output models by Kappel and Munir (37)]. Another potential study for future work could involve estimating model parameters from synthetic timecourse data, to see whether more frequent sampling or a longer observation period provides more stable parameter estimates. However, as highly different parameter values produce similarly excellent fits to the data, the type of data needed for robust parameter estimation is likely to be of a very high quality. As the data are obtained by taking blood samples, there is a practical limitation on the sampling frequency for an individual subject.

The data used were obtained from tracer experiments that were performed between 1963 and 1990. More recent IgG timecourse data are available; however, these data pertain to therapeutic monoclonal antibodies, which can have different kinetics (38). Timecourse data are also available for patients with IgG multiple myeloma, whose serum IgG concentration is monitored during therapy. However, the production rate of IgG in these patients is determined by the status of the disease. Using these data to estimate model parameters would therefore require simultaneous estimation of IgG production parameters. This would require a more complex structural identifiability analysis

and may be considered in future work. For these reasons, more recent data were not used in this study.

The structural identifiability of the relationship between FCR_E and the quantity of endogenous IgG in plasma, $x_{1,E}$, was analyzed. We found that the parameter k_{31} and newly defined parameters $\psi_1 = (k_{03}v_3(k_{14}+k_{off}))/k_{on}$ and $\psi_2 = k_{14}R_{tot}$ are structurally globally identifiable. These new parameters were estimated using least squares estimation. Estimation with synthetic data shows that these parameters can be estimated with a reasonable level of variability. The parameters k_{31} and ψ_2 are physiologically meaningful: k_{31} is the rate at which plasma IgG is internalized into intracellular endosomes and ψ_2 is the maximal rate of recycling of IgG from endosomes into plasma. The 95% confidence interval for k_{31} (0.135–0.174 day^{-1}) is similar to other values reported in the literature [0.13 day^{-1} (17); 0.18 day^{-1} (21); 0.16 day^{-1} (33)]. The 95% confidence interval for ψ_2 (12.3–39.2 μmol day^{-1}) is smaller than previously reported values [68.6 μmol day^{-1} (16); 103 μmol day^{-1} (17)]; however it overlaps with the 95% confidence interval (19.1–60.9 μmol day^{-1}) reported by Kendrick et al. (33).

In applications in which the behavior of the variables $x_3(t)$ and $x_4(t)$, representing unbound and bound IgG in intracellular endosomes, respectively, are of great importance, clearly parameter values are required which determine their behavior, including receptor-ligand binding (k_{on}/v_3, k_{off}, and R_{tot}), recycling of bound IgG into plasma (k_{14}) and degradation of unbound IgG (k_{03}). The results presented in this paper suggest that it is not possible to estimate these parameters from the available data that are only based upon measurements in plasma. In section 3.3, it is shown that these parameters can be varied by several orders of magnitude (see **Figure 8**) whilst having a minimal effect on the plasma IgG response (see **Figure 9A**). It is possible that the actions of the parameters determining recycling, degradation, association and dissociation can approximately balance each other out with respect to the dynamics in the plasma compartment, even though the responses of IgG in the endosome are affected by changes in these parameter values. For investigations limited to the behavior of IgG in plasma, model reduction using the parameters k_{31}, ψ_1, and ψ_2 could be investigated in future work. A two-compartment model based upon the newly derived expression for the FCR has been proposed in section 3.3. Further analysis of this model is required to determine whether it is suitable for investigating IgG responses under a range of clinical conditions.

In future work the models studied in this paper could be used to simulate plasma IgG responses in clinical applications, such as the bone marrow cancer multiple myeloma, in which malignant plasma cells secrete large quantities of monoclonal Ig (M-protein). It has been suggested that the FcRn-IgG interaction may play a significant role in the detection of M-protein using a recently-developed mass spectrometry-based method (4). It was found that in patients with IgG-producing disease, the test result was more likely to be positive for M-protein after three months than in patients with IgA-producing disease, whereas after 12 months the patients were equally likely to have a positive test result. Mills et al. (4) have suggested that this effect is due to FcRn-mediated recycling extending the half-life of IgG, emphasizing the importance of assessment times of response. FcRn-mediated recycling also plays a role in the pharmacokinetics of the novel monoclonal IgG agent for multiple myeloma, daratumumab. Yan et al. (5) found that the isotype of the patient's M-protein has an effect on drug exposure, with IgG patients having significantly lower daratumumab concentrations than patients with other M-protein types. Yan et al. (5) proposed that competition between the IgG M-protein and IgG-based daratumumab for FcRn receptors is the reason for this phenomenon. These recent studies show the importance of FcRn-mediated recycling of IgG in multiple myeloma and the need for mathematical modeling and simulation of this system. The model studied in this paper could be used in future work to investigate such problems.

There is a trade-off in modeling between model accuracy, which is more often represented in complex physiologically-based pharmacokinetic models, and accuracy of parameter values, which is more easily achieved with simplified compartmental models. At present, there are very few studies available on parameter estimation for models of IgG-FcRn kinetics using human data due to issues of parameter identifiability. This paper not only provides useful parameter estimates and suggests a novel model structure, but also exposes some of the difficulties in achieving this aim. Researchers pursuing physiologically-based models of IgG in the future may find it useful to compare the rate of IgG internalization into endosomes and the maximal rate of IgG recycling in their model with the values that we have estimated from human data [considering the approach of Li et al. (34) discussed above]. Furthermore, our paper shows the level of analysis (including structural identifiability analysis, estimation from synthetic data, for example) required in order to have confidence in parameter estimates obtained and an understanding of their meaning to the model.

5. CONCLUSION

It is not possible to estimate all of the model parameters robustly; however certain structurally identifiable parameter combinations have been estimated with a good level of variability. Plasma IgG responses, under typical clinical conditions, are insensitive to large changes in many of the model parameters, provided that certain parameters and parameter combinations are fixed. A reduced-order model, based upon the newly derived expression for the FCR, shows potential for simulating plasma IgG responses under clinical conditions.

AUTHOR CONTRIBUTIONS

FK performed model analyses. FK, MC, and NE wrote the manuscript. SH initiated the work. MC, NE, OB, and SH supervised the work. SH and OB provided discussion on the clinical application of the work. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00674/full#supplementary-material

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Potent Fc Receptor Signaling by IgA Leads to Superior Killing of Cancer Cells by Neutrophils Compared to IgG

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Antibody therapy of cancer is increasingly used in the clinic and has improved patient's life expectancy. Except for immune checkpoint inhibition, the mode of action of many antibodies is to recognize overexpressed or specific tumor antigens and initiate either direct $F(ab')_2$-mediated tumor cell killing, or Fc-mediated effects such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity/phagocytosis (ADCC/P) after binding to activating Fc receptors. All antibodies used in the clinic are of the IgG isotype. The IgA isotype can, however, also elicit powerful anti-tumor responses through engagement of the activating Fc receptor for monomeric IgA (FcαRI). In addition to monocytes, macrophages and eosinophils as FcαRI expressing immune cells, neutrophils are especially vigorous in eliminating IgA opsonized tumor cells. However, with IgG as single agent it appears almost impossible to activate neutrophils efficiently, as we have visualized by live cell imaging of tumor cell killing. In this study, we investigated Fc receptor expression, binding and signaling to clarify why triggering of neutrophils by IgA is more efficient than by IgG. FcαRI expression on neutrophils is ∼2 times and ∼20 times lower than that of Fcγ receptors FcγRIIa and FcγRIIIb, but still, binding of neutrophils to IgA- or IgG-coated surfaces was similar. In addition, our data suggest that IgA-mediated binding of neutrophils is more stable compared to IgG. IgA engagement of neutrophils elicited stronger Fc receptor signaling than IgG as indicated by measuring the p-ERK signaling molecule. We propose that the higher stoichiometry of IgA to the FcαR/FcRγ-chain complex, activating four ITAMs (Immunoreceptor Tyrosine-based Activating Motifs) compared to a single ITAM for FcγRIIa, combined with a possible decoy role of the highly expressed FcγRIIIb, explains why IgA is much better than IgG at triggering tumor cell killing by neutrophils. We anticipate that harnessing the vast population of neutrophils by the use of IgA monoclonal antibodies can be a valuable addition to the growing arsenal of antibody-based therapeutics for cancer treatment.

Keywords: IgA, immunotherapy, neutrophil, ADCC, cancer, Fc alpha receptor I, CD89, signaling

INTRODUCTION

Antibody therapy for cancer treatment is increasingly used in the clinic after the successful introduction of rituximab over two decades ago, which is an IgG monoclonal antibody (mAb) directed against CD20 expressed on B cells. This success has been followed by the development of many IgG-based mAbs for cancer treatments and has considerably improved treatment outcome. These mAbs can employ direct working mechanisms through their $F(ab')_2$ domains by interfering with target function or inducing complement-dependent cytotoxicity (CDC) after binding of C1q to clustered Fc domains on the tumor cell surface. Next to CDC, binding of the IgG Fc domain to Fcγ receptors (FcγR) expressed on immune cells can elicit antibody dependent cell-mediated cytotoxicity/phagocytosis (ADCC/P). For antibody therapy in humans, it remains difficult to assess the contribution of each working mechanism for different mAbs, but *in vivo* experiments have exposed an important contribution of Fc receptor-mediated ADCC/P (1, 2). In addition, the role of FcγR in humans has been further demonstrated by genetic polymorphisms of FcγR that influence clinical outcome of mAb therapy (3).

All the current therapeutic mAbs for cancer are based on the IgG isotype. Reasons for this include its natural prevalence in the body, long half-life of IgG, and the substantial amount of fundamental and biotechnological knowledge of this isotype. IgG mAbs that trigger ADCC/P are described to activate NK cells by FcγRIIIa and monocytes/macrophages by the various activating FcγRs they express. Activating FcR signal via ITAMs (Immunoreceptor Tyrosine-based Activating Motifs), either in their cytoplasmic domain or via the FcR-associated gamma chain. Upon antibody binding and crosslinking of FcR, ITAMs will first bind and activate Lyn and/or Fyn tyrosine kinases, depending on the immune cell. Subsequently, phosphorylated ITAMs will recruit and activate Syk followed by the activation of SOS, Ras, Rac, PKC, PI3K, and finally ERK or MAP kinase, inducing gene transcription of cytokines, inflammatory mediators, microbicidal enzymes, activation of the cytoskeleton, all together leading to ADCC, phagocytosis, cell migration, and degranulation. These pathways are comparable between different activating Fc receptors for different Ig isotypes (4). Recent discoveries advocate that other isotypes, like IgA and IgE, are also promising options for tumor treatment (5, 6).

IgA directed against tumor cells has been proven to be effective *in vivo*, which largely depends on the presence of FcαRI, the myeloid Fc receptor for monomeric IgA (7–11). FcαRI is expressed by innate immune cells, including monocytes, macrophages, Kupffer cells, eosinophils, and neutrophils (12, 13). Neutrophils are the most abundant immune cells in the body, representing up to 70% of circulating leukocytes. They migrate through and surveil tissues, including malignant tumors, and can mediate antibody-induced anti-tumor effects (14, 15). Neutrophils are superior at eliminating IgA-opsonized tumor cells compared to IgG using EGFR, CD20, HER2 and HLA II as targets in *in vitro* studies (7, 9, 10, 16–20). In addition, *in vivo* studies using intraperitoneal tumor models indicate that neutrophils are recruited to the peritoneum upon IgA treatment (unpublished data). Neutrophils can exert several effector functions, including phagocytosis, degranulation, ROS production and NET formation (21). These effector mechanisms are found not to be causal for killing of opsonized tumor cells. Recently, a new neutrophil effector mechanism, termed trogoptosis, has been characterized (22, 23). Trogoptosis is target cell death initiated by antibody-FcR-triggered trogocytosis (24) executed by stimulated neutrophils and has also been described as ADCT (antibody-dependent cell-mediated trogocytosis). A similar process has been postulated to be executed by macrophages but this requires more time (25).

To improve antibody therapeutics, it would be promising to mobilize the vast number of neutrophils with IgA to help eradicate tumor cells from the body. In the current study we investigated the underlying mechanisms of the superior IgA-mediated tumor cell killing by neutrophils in comparison to IgG. First, IgA and IgG were compared in classical ^{51}Cr release assays using three clinically used targets to verify published observations. To visualize the IgA-mediated killing by neutrophils, we performed live-cell imaging in a similar setup. An explanation for the strong ability of IgA to efficiently induce killing by neutrophils could be a correlating difference in the expression of FcγRs and FcαRI. Therefore, the quantitative FcR expression level on neutrophils and their binding capacity to IgG- and IgA-coated or -opsonized surfaces were explored. Antibody Fc domain binding to FcR facilitates its ITAM phosphorylation and ultimately the phosphorylation and activation of ERK. Therefore, we also investigated the dynamics of p-ERK in neutrophils after interaction with IgA- and IgG-bound surfaces, and found significant differences.

MATERIALS AND METHODS

Reagents, Antibodies

Antibodies: to target human CD20, rituximab (Roche), anti-CD20-IgA1 (invivogen, hcd20-mab6), or in-house made mAbs (26) were used. For HER2, anti-HER2 IgG1 antibody trastuzumab (Roche), anti-HER2 IgA1 or IgA2 antibody was used and for EGFR, anti-EGFR IgG1 antibody cetuximab (Merck) or anti-EGFR IgA2 was used. Anti-HER2 or EGFR IgA's were made as described before (11). 3G8 $F(ab')_2$ (anti-FcγRIII) was generated by pepsin protease procedure of purified 3G8 from hybridoma supernatant. Rabbit anti-ERK (9101S) and rabbit anti-p-ERK (9102S) both from Cell Signaling Technology, anti-β-Actin (Sigma-Aldrich, A2228), anti-rabbit IgG HRP (Santa Cruz, sc-2004), goat anti-mouse IgG HRP (Santa Cruz, sc-2005), Strep-Tactin HRP (Bio-Rad Laboratories, 16-10380) were used for Western blot detection. The Qifikit (DAKO) was used to determine the number of FcγRs on primary neutrophil isolates using anti-CD64 (clone 10.1, Serotec), anti-CD89 (clone A59, BD Pharmingen), anti-CD32a (clone IV.3, Stemcell Technologies), anti-CD16 (clone 3G8, Stemcell Technologies), and anti-CD32a/b (clone AT10, Santa Cruz Biotechnology or clone KB61, DAKO). Pharmacological compounds Wortmannin (Sigma-Aldrich), LY294002, and U0126 (Calbiochem) were used to inhibit ITAM signaling in ^{51}Cr release assays. Calcein-AM (Life Technologies) was used to fluorescently label target cells

or neutrophils according to the manufacturers protocol. TO-PRO^{TM}-3 (molecular probes) was used at 1:1000 dilution to detect DNA that becomes accessible during live-cell imaging (**Figure 1E**, **Videos S1**, **S2**).

Primary Neutrophils and Cell Lines

Neutrophil cell fraction was isolated from blood of healthy donors (in agreement with ethical committee of the Utrecht university medical center and after written informed consent from the subjects in accordance with the Declaration of Helsinki) using standard Ficoll/Histopaque density block gradient centrifugation (Ficoll-paque was from GE healthcare, ref. 17-1440-03, Histopaque-1119 was from Sigma-Aldrich ref. 11191) followed by lysis of the red blood cells in ammonium buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.037 mg/mL Na_2EDTA, pH 7.4) for 10 min on ice. Neutrophils were preserved in complete medium (RPMI 1640 from Gibco supplemented with glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin from Life Technologies) before use on the same day unless stated otherwise. EL4-CD20, Ba/F3-HER2, and A431-HER2 cells (ATCC) were generated by retroviral transduction as described before (18). Ramos, Daudi, SK-BR-3, A431 (ATCC), and the above-mentioned cells were cultured in complete medium at 37°C and 5% CO_2.

Live-Cell Imaging

For live-cell imaging, a Deltavision RT widefield microscope (GE Healthcare) equipped with a conditioned imaging chamber set to 37°C and 5% CO_2 was used. Time-lapse imaging was performed using an Olympus 40×/1.35 NA (numerical aperture) oil immersion objective (**Figure 1E**, **Videos S1, S2**) or an Olympus 20×/0.75 NA objective (**Figure S1**, **Videos S3–S5**) and images were recorded on a Cascade II EM-CCD camera (Photometrics). For **Videos S1**, **S2**, the A431 cells were seeded in a 6-channel μ-slide (Ibidi) the day before calcein labeling and live cell imaging. Target cells in **Videos S3–S5** were harvested and cytosolically labeled with calcein-AM and allowed to interact with primary neutrophils together with mAbs for up to 2 h in the 6-channel μ-slide. Image acquisition (30–60 s between frames) started as soon as possible upon addition of the mAbs. Imaging data was processed using SoftWoRx (AppliedPrecision) or Imaris (Bitplane).

Human Neutrophil ADCC

ADCC with ^{51}Cr-labeled target cells was described previously (18). Briefly, 1×10^6 target cells were labeled with 100 μCi (3.7 MBq) ^{51}Cr for 2 h in complete medium. After extensive washing, cells were adjusted to 10^5/mL. Neutrophils, mAbs at various concentrations, medium, and 5,000 tumor cells per well were added to round-bottom microtiter plates (Corning Incorporated) using a maximum E:T = 40:1 ratio. When indicated, neutrophils were preincubated for 15 min at RT with inhibitors of signaling molecules before they were added to the plate. After 4 h of incubation at 37°C, ^{51}Cr release was measured in counts per minute (cpm). The percentage of specific lysis was calculated using the following formula: % lysis = [(counts of sample–minimum release)/(maximum release–minimum release)] × 100. Target cells with neutrophils in complete medium or supplemented with 5% Triton X-100 (Roche Diagnostics) were used to determine minimum and maximum release, respectively.

Binding of Neutrophils on Antibody-Coated Plastic Surface

For binding assays in 96-wells flat bottom maxisorp plates, wells were coated with 100 μL 10 μg/mL antibody in carbonate buffer (Sigma, C3041-50CAP) O/N at 4°C. Plates were washed with 100 μL complete culture medium, blocked for 1 h with 1% BSA and washed again with culture medium before adding the cells. Neutrophils were isolated from blood and labeled with 10 μM Calcein-AM in PBS at 37°C for 20 min. Cells were washed two times with complete medium and rested for 30 min at 37°C at a density of 3×10^6 cells/mL. 100 μL neutrophil suspension was added per well and centrifuged gently (∼50×g) for 3 min. The plate was incubated at 37°C for 40 min. Fluorescence was measured in a Clariostar fluorescence scanner. After measurement, the plate was washed with 100 μL RPMI. Measurements were repeated every 2 washes and this was repeated at least 12 times.

Bead Binding to Neutrophils (Rosettes)

The rosette assay using Dynabeads was adapted from a previously described protocol (27). Epoxy-Dynabeads (4.5 mm) were coupled to either anti-CD20 mAbs UMAB002 IgG1, UMAB002 IgA2, anti-HER2 mAbs trastuzumab (Herceptin) IgG1, anti-HER2 IgA2 (own production), or human serum albumin (Albuman®, Sanquin) following the manufacturer's instructions (Thermo Fisher Scientific). Labeling of the beads was checked with RPE-labeled anti-IgA (Southern Biotech, 2052-09), and PE-labeled anti-IgG (Southern Biotech, 2042-09). To allow bead binding, 50 μl 2×10^6 cells/mL neutrophils in ice-cold PBS containing 0.5% BSA were pipetted in a round bottom 96-wells plate (Greiner) on ice. 50 μL 1×10^7 beads/mL bead suspension in PBS containing 0.5% BSA was added to the cells. Beads were either coated with anti-CD20 or anti-HER2 IgG1 or IgA2. Plates were incubated on a shaker (800 RPM) at 4°C for at least 30 min. The plate was then incubated at 37°C for 10 min and immediately put on ice. After centrifugation and supernatant removal, the samples were fixed in 3% PFA in PBS containing 0,5% BSA for 15 min at RT. The fixed cell/bead mixture was transferred to a flat-bottom 96-wells plate, centrifuged (∼50×g) for 3 min and images were taken with brightfield microscopy (EVOSRXLCore) and analyzed using Adobe Photoshop using raster blocks of 11 × 11 cm. Cells were counted manually and the percentage of cells bound to 5 or more beads was calculated per image, for 3 images per condition.

Real-Time Tracing of Antibody-Mediated Cell-Cell Interactions

Daudi cells were adhered in quarters A&C of LigandTracer Multidishes 2 × 2 (non-treated, Ridgeview Instruments AB) with the help of a biomolecular anchor molecule (SUNBRIGHT® OE-040CS, NOF Corporation), essentially as previously described (28). Per immobilization spot, 400 μl BAM solution (4 mg/ml, dissolved in MQ water) was incubated for 1 h at room temperature and after removal of the BAM solution 400 μl cell suspension (1.5×10^6 cells/ml in PBS) was added and cells

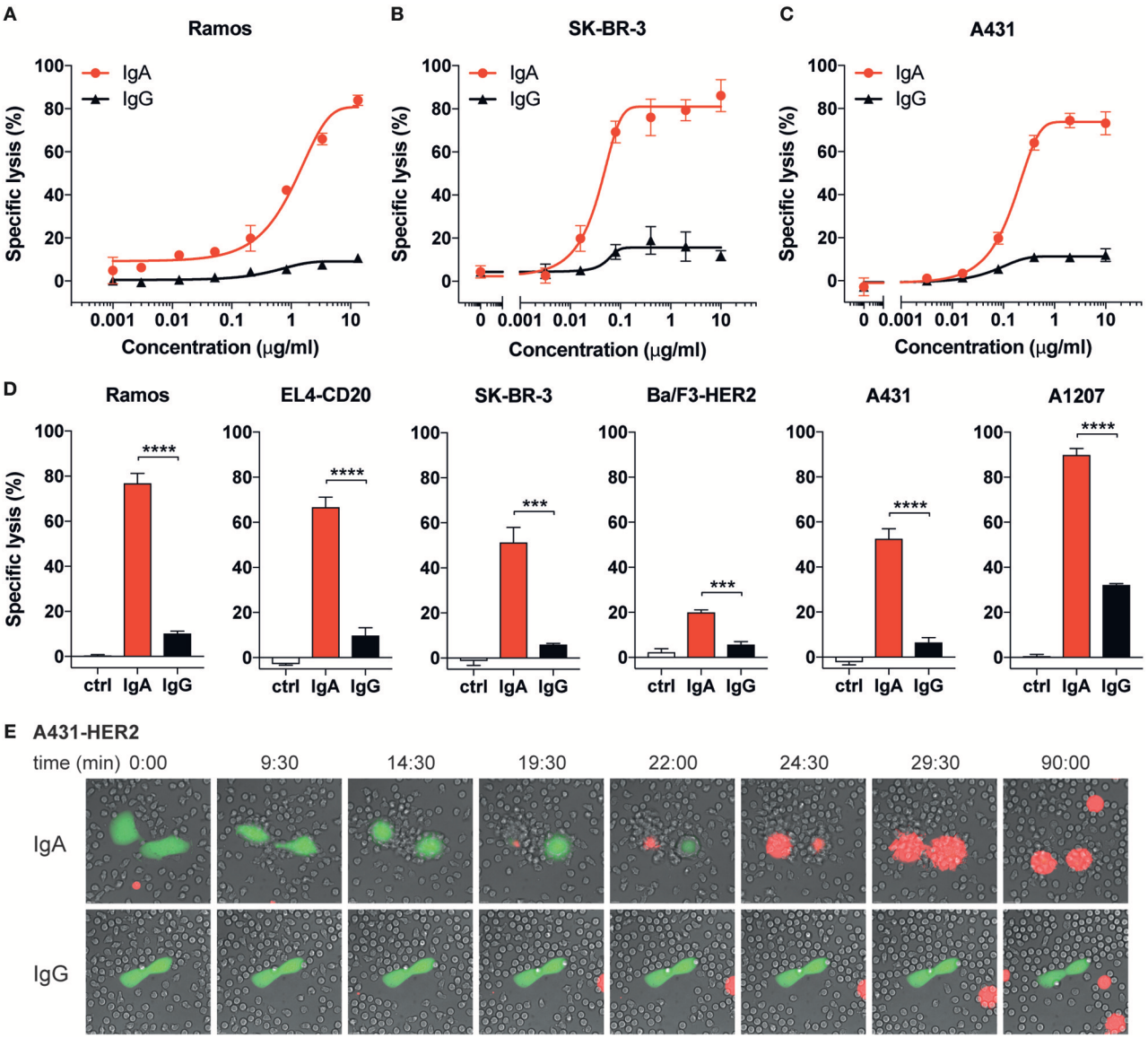

FIGURE 1 | IgA mediates higher tumor cell lysis than IgG with human neutrophils. **(A–D)** Specific lysis of tumor cells by isolated human neutrophils (E:T = 40:1) using a 4 h ^{51}Cr release assay. Anti-CD20 Abs were used for Ramos and EL4-CD20, anti-HER2 Abs were used for SK-BR-3 and Ba/F3-HER2, anti-EGFR Abs were used for A431 and A1207 target cells. **(A–C)** Specific lysis of indicated target cells using a broad titration range of IgA and IgG antibodies. **(D)** Antibody concentrations were identical (10 μg/mL) for IgG and IgA antibodies against all target cells except for Ramos (13.3 μg/mL) and A1207 cells (1 μg/mL). Ctrl indicates condition without antibody (or 0.001 μg/mL IgG1-anti-CD20 for Ramos cells). One representative graph is shown for $n = 4$–6 independent experiments with different healthy donors. $p < 0.001$: ***, $p < 0.0001$: ****, unpaired Student's t test. **(E)** Stills from live cell microscopy of adhered A431-HER2 cells (calcein labeled) and neutrophils in the presence of 5 μg/mL anti-HER2 IgA2 or IgG (Trastuzumab) and TO-PRO-3 (red fluorescence), time in minutes, image acquisition every 30 s for 1.5 h.

were left to adhere for 40 min. Prepared cell dishes were kept in complete medium in the incubator and used for experiments the following day. For measuring antibody mediated cell-cell interactions, one of the immobilized Daudi cell spots was preincubated with 50 nM of either anti-CD20 IgG1 or IgA2 (own production, clone UMAB001) for 1 h in LigandTracer Green (Ridgeview Instruments AB) at room temperature. Antibody preincubation was done only for one of the compartments, the other half of the dish served as control for quantifying non-antibody mediated neutrophil interactions. To each half of the dish, 3×10^6 calcein-labeled neutrophils from the same donor were added and binding to Daudi cells was recorded once every 75 s. After 1 h, neutrophils were removed and the remaining cell-cell complexes were followed for another hour to observe their stability. Binding slopes from the no antibody control were used to normalize the data for differences in signal height between experiments. The binding association between IgG1 and IgA2 was compared by the ratio of the binding

slope, which was calculated with TraceDrawer 1.8 (Ridgeview Instruments AB). The stability of the formed cell-cell complexes is represented by the half-life, which was calculated from the dissociation rate constant k_d that was obtained by fitting a single exponential decay to the dissociation phase (TraceDrawer 1.8).

Neutrophil Stimulation With Antibody-Coated Beads and Western Blotting

Neutrophils were brought to 1×10^8 cells/ml in Hanks buffer. Per condition, neutrophils were stimulated with antibody-coated beads in a 1:4 = neutrophil:bead ratio at 37°C for the indicated time periods. After incubation, cells were lysed in Laemmli reducing sample buffer and boiled. SDS-PAGE (12%) was performed and gels were blotted on nitrocellulose membranes. For ERK/p-ERK detection, the membranes were first blocked in 4% ELK milk in TBST (TRIS buffered saline/0.1% Tween 20), washed in TBST and incubated with anti-ERK or anti-p-ERK both at 1:2500 in TBST containing 0.3% BSA at 4°C overnight. Then, blots were washed in TBST, blocked in 4% ELK milk in TBST for 1 h at RT and sequentially incubated with anti-rabbit IgG HRP (1:2500) and Strep-Tactin HRP (1:5000) in 1% ELK milk in TBST for 1 h at RT. Blots were sequentially washed with TBST and PBS followed by ECL-based detection in a BioRad Gel Doc. For the β-actin immuno staining, the HRP from the former detection was first destroyed by 24 h incubation at 4°C in PBS containing 0.1% Tween 20, 0.6% sodium azide, and 0.6% H_2O_2. This solution was refreshed at least three times during incubation. After this, the blots were washed with TBST, blocked for 1 h at RT in 4% ELK milk in TBST followed by 2 h incubation with anti-β-Actin (1:2000) in 1% milk in TBST. The blots were then washed with TBST and incubated for 1 h with goat anti-mouse IgG HRP (1:5000) in 1% milk and further processed for detection like described above.

Statistical Analysis

Graphs represent mean ± SD. Statistical analysis was performed by using unpaired Students *t*-tests or ANOVA with Tukey's multiple comparison test, $p < 0.05$ were considered as statistically significant.

RESULTS

IgA Mediates Superior Neutrophil Cytotoxicity on Tumor Cells Compared to IgG

We and others have demonstrated that IgA-opsonized tumor cells, in contrast to IgG, are efficiently killed by freshly isolated neutrophils (7, 9, 10, 16–20). To verify previous observations, we performed ^{51}Cr release assays using three targets (CD20, HER2, and EGFR) and freshly isolated unstimulated neutrophils from healthy donors (**Figure 1**). The mAbs were first titrated in a broad concentration range and this shows that, although the antibody concentration for IgA and IgG for detectable lysis is similar, the highest maximum amount of lysis is achieved by IgA (**Figures 1A–C**). We further inventoried this with two cell lines per target, confirming that the IgA isotype consistently outperforms IgG in tumor cell lysis by primary unstimulated neutrophils (**Figure 1D**). The magnitude of lysis did vary between mAbs, cell lines and neutrophil donors, but the superiority of IgA was found to be highly constant in this setting.

To visualize the process of antibody-mediated tumor cell killing by neutrophils, we performed live-cell imaging. In the presence of anti-HER2 IgG (Trastuzumab), A431-HER2 cells did not elicit a clear recruitment of unstimulated primary neutrophils and only minimal interaction between A431-HER2 and neutrophils was observed (**Figure 1E** lower panel and **Video S2**). In contrast, neutrophils rapidly respond to A431-HER2 in the presence of anti-HER2 IgA (**Figure 1E** upper panel and **Video S1**). The fast reaction of neutrophils suggests a swarming effect toward the calcein-labeled tumor cell and involves numerous interactions which seem reminiscent of trogocytosis (23). Only in the presence of IgA, the tumor cells succumb to these events within 30 min as indicated by the fluorescence of a DNA-binding dye. The killing process of IgA-opsonized cells was also visualized for EGFR- and CD20-expressing target cells and demonstrated similar dynamics between neutrophils and tumor cells. Several lysis events are observed with IgA (**Figures S1A,B**, **Videos S3–S5**) whereas with IgG no lysis could be recorded (data not shown). These data confirm the superiority of IgA over IgG in neutrophil-mediated killing of tumor cells for three different targets and seems to involve the recently described trogoptosis as an antibody-dependent cytotoxic working mechanism of neutrophils.

Differential FcR Expression on Neutrophils Does Not Explain IgA Superiority

To find a possible explanation for the robust IgA-dependent tumor cell killing, we quantified FcαRI expression on unstimulated primary neutrophils. A relatively high expression of FcαRI would possibly explain the stronger activation of neutrophil effector mechanisms after engagement with IgA-opsonized cells. However, our analysis of the number of FcR molecules per neutrophil demonstrated that the expression of FcαRI is actually ∼2-fold lower than that of FcγRIIa, the activating FcγR on neutrophils (**Figure 2A**). FcγRI and FcγRIIIa are not detectable on unstimulated neutrophils (29) and the GPI-linked FcγRIIIb is expressed at least 10-fold higher than FcγRIIa and FcαRI. The high constitutive expression of FcγRIIIb is unique for neutrophils as this is not observed on monocytes or NK cells (**Figure S2**) and other granulocytes (29).

Neutrophils do not express an ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif)- containing FcγR that could counteract signaling by activating FcγRs. Therefore, we continued by investigating the potential role of FcγRIIIb that has been reported to interfere with Fc-mediated effects, although it is a GPI-linked protein and cannot signal on its own (30–32). Blocking FcγRIIIb with 3G8 mAb F(ab′)$_2$ in a ^{51}Cr-release assay did, however, only marginally improve IgG-mediated lysis of tumor cells (**Figure 2B**) and did not change with 10 times higher 3G8 concentration (data not shown). For all three antibodies tested, IgA outperformed IgG even after FcγRIIIb blockade. From these data we conclude that neither the FcR expression levels nor a major role for FcγRIIIb in inhibiting

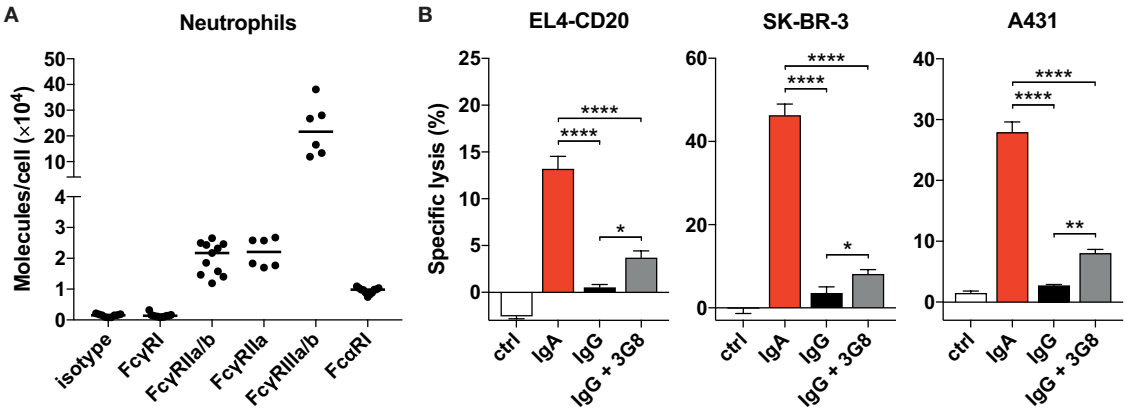

FIGURE 2 | Blocking FcγRIIIb only marginally improves tumor cell lysis. **(A)** Quantitative expression of FcγR and FcαRI on human neutrophils as analyzed by flow cytometry (Qifikit), $n = 6$–11 healthy donors. **(B)** 3 h ^{51}Cr release assays using EL4-CD20 with anti-CD20 IgA2 or IgG1 (rituximab), SK-BR-3 with anti-HER2 IgA1 or IgG1 and A431 with anti-EGFR IgA2 or IgG1, all at 10 ug/mL. 3G8 F(ab′)$_2$ fragments (1 ug/mL final concentration) were added 15 min prior to start of the ADCC assays. Statistics: one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$: *, $p < 0.01$: **, $p < 0.0001$: ****. Ctrl indicates no antibody added.

FcγRIIa function are causal for the discrepancy between IgA- and IgG-mediated killing by primary neutrophils.

Neutrophils Have Similar Binding Characteristics to IgA- or IgG-Bound Surfaces

Efficient IgA-induced killing by neutrophils could also be driven by stronger binding to its FcR compared to IgG. The binding affinities of monomeric IgA to FcαRI and monomeric IgG to FcγRIIa and FcγRIIIb are in the low affinity range with reported K_a of $<10^7$ M^{-1} and therefore believed to be short-lived (29, 33). Stable binding is only achieved when sufficient avidity is established by an increase in valency from the multiple interactions of FcRs to antibody-bound surfaces. Therefore, we interrogated the binding characteristics of primary neutrophils to antibody-coated or -opsonized surfaces. Unstimulated primary neutrophils from healthy donors were labeled with calcein, allowed to bind antibody-coated surfaces and subjected to several wash steps while monitoring calcein fluorescence in the wells (**Figures 3A–D**). Often, higher binding of neutrophils to IgA-coated surfaces was measured (**Figure 3A**), but, depending on donor and mAb, the reverse was also found (**Figures 3B,D**). Only for the HER2 target a consistent better binding to the IgA isotype was observed with the used donors.

We continued by performing rosette assays where neutrophils are exposed to IgA- or IgG-coated beads (**Figures 3E–G**). The example images shown in **Figure 3E** reveal that more neutrophils bind IgA-coated beads. Further analysis with either anti-CD20- or anti-HER2-coated beads showed a trend for more rosettes and thus better binding with IgA compared to IgG, but this did not reach statistical significance (**Figures 3F,G**).

Binding dynamics of neutrophils to opsonized target cells was also explored with LigandTracer technology (28). Calcein-labeled primary neutrophils were first allowed to engage anti-CD20 IgA- or IgG-opsonized Daudi cells as illustrated by the increase in fluorescent signal (**Figure 3H**). After removal of the unbound neutrophils, the remaining neutrophils were monitored further in time to study the stability of the neutrophil-Daudi interaction. This approach confirmed our previous observations that there is no significant difference between IgA- and IgG-mediated binding of neutrophils (**Figure 3I**). We did, however, notice an increased stability of the Daudi:neutrophil complex (**Figure 3J**, **Figure S3**) when IgA was used, although significance is not reached. Taken together, binding of neutrophils to IgA bound surfaces is at least as good as with IgG despite the lower expression of FcαRI compared to the FcγRs.

IgA Elicits Stronger ITAM Signaling in Neutrophils Than IgG

Thus far, a clear mechanism that accounts for the superior IgA- vs. poor IgG-mediated killing by neutrophils has not been identified. For neutrophils to exert their effector functions after FcR binding, ITAM signaling is required. Therefore, we measured the magnitude of signaling in neutrophils after binding to antibody-coated surfaces. Analysis by immunoblotting revealed a rapid and strong phosphorylated ERK (p-ERK) signal after 5 min when neutrophils were allowed to interact with IgA-coated beads, while IgG-coated beads induced only a weak p-ERK signal (**Figures 4A–D**). These conditions were repeated with several neutrophil donors for both anti-CD20 and anti-HER2 antibodies, demonstrating a reproducible effect on the p-ERK signal (**Figures 4A–D**, **Figures S4A–C**).

To confirm the crucial role for the robust FcαRI signaling, we tested the PI3K inhibitors wortmannin and Ly294002 and the MEK1/2 inhibitor U0126, which both act upstream of ERK in the signaling cascade. The presence of these pharmacological inhibitors in ^{51}Cr-release assays resulted in a significant decrease in tumor cell lysis (**Figure 4E**). This quantitative difference in signaling and its requirement for efficient antibody mediated killing leads to a model where FcαRI

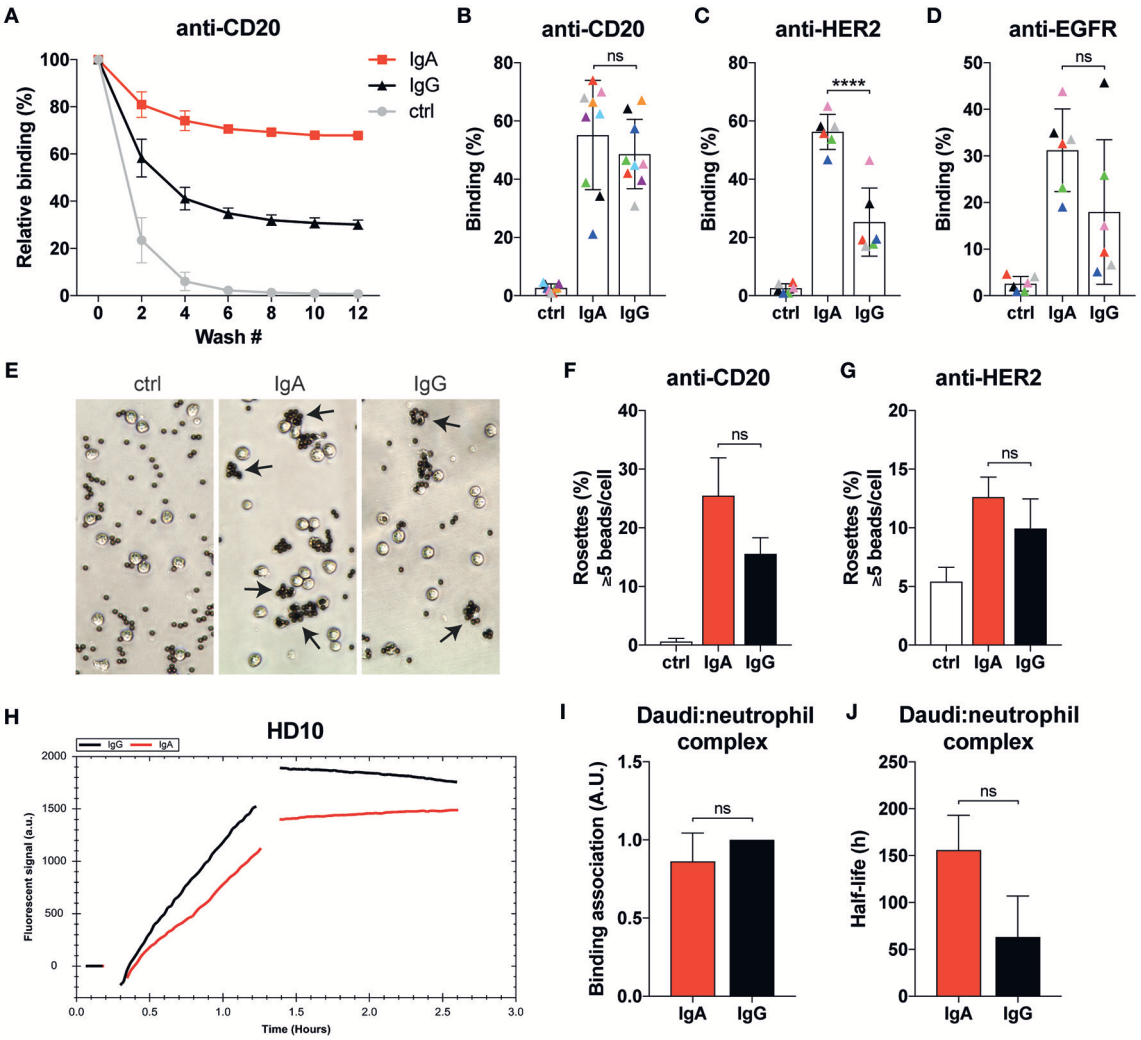

FIGURE 3 | Neutrophils display similar binding characteristics to IgA- or IgG-associated surfaces. **(A)** Example of relative binding of a donor of which calcein labeled neutrophils were allowed to associate to anti-CD20 IgA2 or IgG1 (clone UMAB001) coated 96 well plate. Remaining calcein fluorescence was measured after repeating wash steps. Calcein fluorescence before the first wash was set 100%. Ctrl indicates that no antibody was used during coating. **(B–D)** As in **A**, but data of 6–9 different donors at wash 10 are combined per target being anti-CD20 IgA2 or IgG1 (clone UMAB001) or anti-HER2 IgA2 or IgG1. Each color indicates data from the same donor. Statistics: two-way ANOVA with Tukey's multiple comparison test, $p < 0.0001$: ****. **(E)** Examples images of neutrophils binding to albumin, anti-CD20 IgA, or IgG coated Dynabeads (ratio beads:neutrophils = 5:1). Rosettes are defined as cells binding 5 or more beads. **(F,G)** Quantification of rosettes of anti-CD20- or anti-HER2-coated Dynabeads with neutrophils. One representative of $n = 3$ individual experiments is shown. Differences were not significant according to one-way ANOVA with Tukey's multiple comparison test. **(H)** Binding traces for calcein labeled neutrophils binding to anti-CD20 IgA- (red) or IgG- (black) opsonized Daudi cells. One representative of $n = 5$ experiments is shown. **(I)** Binding association and **(J)** average half-life of the Daudi:neutrophils complex pooled from 5 different donors. Statistics: paired Student's *t*-test.

signaling is stronger than FcγRIIa signaling in neutrophils, which can explain the efficient killing of IgA-opsonized tumor cells (**Figure 5**).

DISCUSSION

FcR-bearing immune effector cells have a prominent role in antibody therapies for cancer treatment by engaging Fc domains of IgG-opsonized tumor cells. Due to the IgG based format of these therapeutics, only FcγR-mediated ADCC, ADCP, and/or ADCT mechanisms can be employed by immune cells. Our data confirms that unstimulated neutrophils are very poor at eliciting lysis of tumor cells through the IgG-FcγR axis. Yet, it could be very propitious if the vast number of neutrophils in the body could be recruited for tumor eradication. In agreement with previous work, we demonstrated for three targets (CD20, EGFR, and HER2) that the IgA isotype is very potent at triggering tumor cell killing by unstimulated neutrophils. Former studies have compared this to IgG-mediated killing by NK cells and demonstrated that the maximal IgA-mediated killing by neutrophils is often higher (7, 10, 18). NK cell-mediated killing by IgG does seem to be more efficient

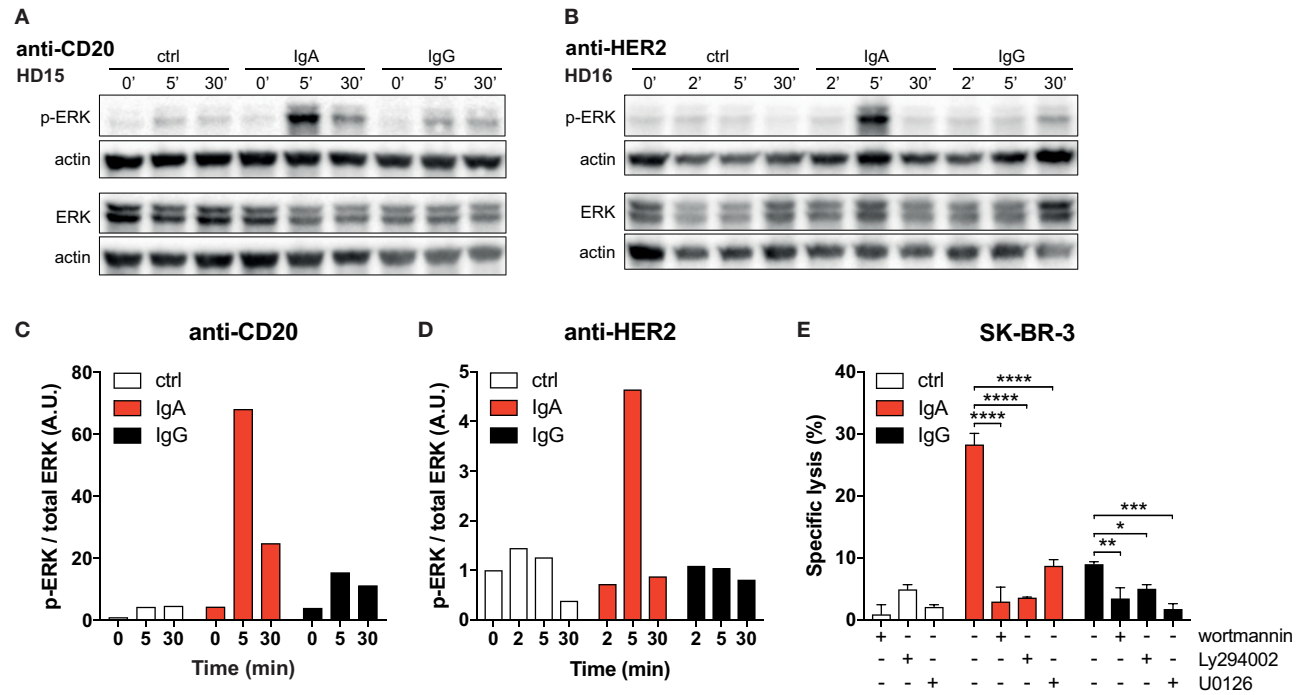

FIGURE 4 | Strong p-ERK signal in neutrophils elicited by IgA. **(A,B)** Time-dependent increase and decrease of p-ERK signal in neutrophils after exposure to anti-CD20 or anti-HER2 IgA2- or IgG1-coated Dynabeads. Actin detection was performed on the same blot after destroying the p-ERK or total ERK signal. **(C,D)** Quantification of signaling defined as the ratio of normalized p-ERK/actin over normalized total ERK/actin. Data was normalized against the 0 min time point. **(E)** 4 h ^{51}Cr release assays using SK-BR-3 with anti-HER2 IgA2 or IgG1, both at 1 ug/mL. Where indicated, pharmacological inhibitors of signaling factors were included (wortmannin at 0.5 μM, Ly294002 and U0126 both at 20 μM). Statistics: two-way ANOVA with Tukey's multiple comparisons test $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***, $p < 0.0001$: ****.

at lower antibody concentrations compared to IgA-induced lysis by neutrophils. Visualization of the IgA-mediated killing process by neutrophils did not reveal signs of phagocytosis, but suggests a killing mechanism that includes frequent and vigorous interactions between neutrophils and IgA-opsonized tumor cells, most likely involving trogocytosis. These observations agree with trogoptosis, a trogocytosis-based mechanism that has been postulated for IgG-mediated killing by stimulated neutrophils (23) and recently submitted work on IgA (Treffers et al. submitted). Neutrophils have been described to display a swarming behavior toward sites of inflammation or tissue damage in which LTB4 secretion is an important molecule (34–36). In our live-cell experiments (**Video S2**), migration of neutrophils toward the IgA-opsonized tumor cell resembles this swarming phenotype that precedes tumor cell death. If such a process could be established in malignant tumors by IgA mAbs, it might break the immune tolerant tumor microenvironment and drive a robust anti-tumor response.

In our quest for the explanation of the superior IgA-mediated tumor cell killing by neutrophils, we investigated its binding dynamics to antibody-bound surfaces, the role of FcγRIIIb and its FcR expression levels. Unexpectedly, FcαRI expression by neutrophils is ∼2-fold lower than that of FcγRIIa. The FcαRI expression level measured on neutrophils does, therefore, not provide a simple explanation for the robust IgA-elicited killing by neutrophils in comparison to the poor IgG-mediated killing. Blocking of the highly expressed GPI-bound FcγRIIIb during a ^{51}Cr-release assay also does not restore IgG-induced tumor cell lysis to the level achieved with IgA. Still, the abundant expression of FcγRIIIb could interfere by preventing optimal FcγRIIa organization at the plasma membrane required for efficient ITAM signaling and triggering of neutrophil effector function. Another reason for the efficient IgA-mediated killing could be a better qualitative and quantitative IgA-FcαRI interaction by neutrophils. We conducted several binding studies using IgA- or IgG-bound surfaces and even opsonized cells as platforms for unstimulated primary neutrophils to bind to (**Figure 3**). Although a trend of better binding for IgA was observed, no clear significant differences for all of the tested targets were found. The very consistent superiority of IgA-mediated lysis does not correlate with the similarity we see in binding to IgA or IgG of neutrophil from different donors. Thus, the characterized binding dynamics do not provide a satisfactory answer for the effectiveness of IgA-induced killing by primary neutrophils.

As binding characteristics cannot explain the poor IgG-mediated killing, we reasoned that it might not be the binding itself that is the crucial factor, but rather the events that happen after binding. Indeed, the magnitude of FcR signaling within neutrophils after exposure to IgA on p-ERK level is

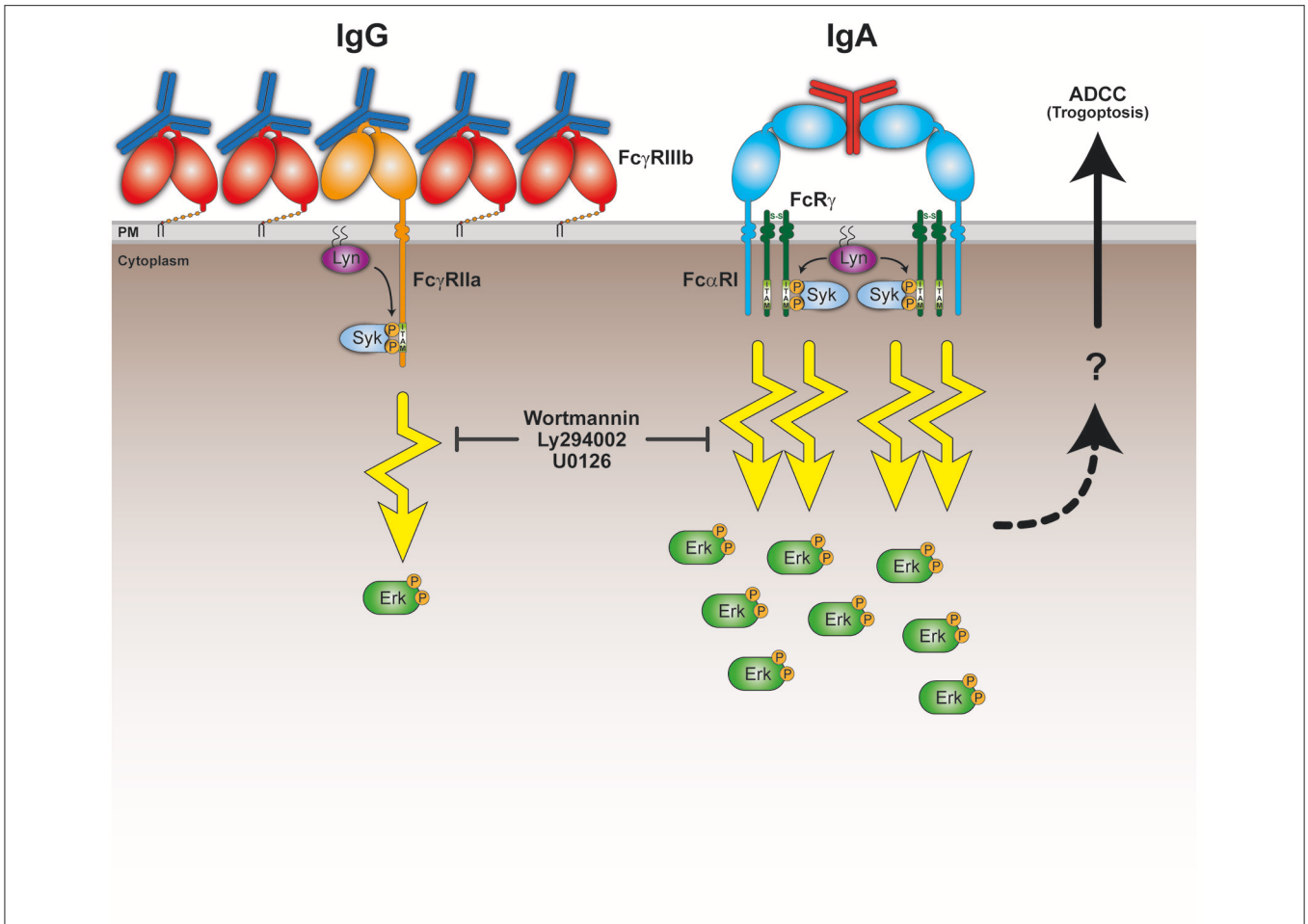

FIGURE 5 | Model for superior IgA-mediated tumor cell killing by neutrophils. Neutrophils express the low affinity Fc receptors FcγRIIIb, FcγRIIa, and FcαRI. Both FcγRIIa and FcαRI are activating Fc receptors, because upon ligand (antibody Fc domain) binding, clustering and recruitment of kinases (e.g., Lyn), they signal through their ITAM domains and elicit cellular effector functions. The relatively high FcγRIIIb expression on neutrophils could possibly interfere with the activating capacity of FcγRIIa by competing for Fc domain binding and preventing proper formation of signaling platforms and/or organization within the lipid bilayer. This results in a poor ability of FcγRIIa to initiate sufficient signaling in neutrophils. Despite the low FcαRI expression on neutrophils, they can still engage clustered IgA Fc domains to a similar degree as IgG Fc domains bind the FcγRs. IgA can, however, bind FcαRI bivalently resulting in more stable binding and recruitment of in total 4 ITAMs. This scenario would initiate a robust ITAM signaling necessary for activating effector functions, including trogoptosis to eliminate tumor cells. This is further illustrated by the signaling inhibitors wortmannin, Ly294002 and U0126 that prevent tumor cell lysis *in vitro*. The question mark refers to yet unclear processes involving signaling, intracellular Ca^{2+}, actin-myosin contraction, and immune cell-tumor cell interactions that lead to ADDC by neutrophils (trogoptosis) (23).

much stronger than for IgG (**Figures 4A–D**). It was confirmed in **Figure 4E** that inhibitors of the ITAM signaling are very effective in preventing IgA-mediated tumor cell lysis (**Figure 4E**). Therefore, very strong ITAM signaling upon FcαRI engagement explains the potent neutrophil effector functions. The relatively low FcαRI expression on neutrophils, but yet similar binding to IgA and its powerful IgA-induced signaling suggests a fundamental difference in the manner of FcR engagement. FcαRI is able to interact with IgA in a 1:1 or a 2:1 (FcαRI:IgA) stoichiometry (**Figure 5**) (37). A bivalent binding of FcαRI to IgA would result in a stronger association, which is supported by our observation that the half-life of IgA-mediated binding tends to be longer compared to IgG (**Figure 3J**). Moreover, FcγRIIa can only signal through one ITAM located in its cytoplasmic tail, whereas FcαRI in a 2:1 stoichiometry would deploy four ITAMs by the FcαRI-associated FcRγ-chains resulting in much stronger signaling (**Figure 5**). It would be interesting to test this model by comparing monovalent vs. bivalent IgA-FcαRI binding. This requires challenging engineering of IgA molecules in which the crucial residue(s) for FcαRI binding (38) are mutated in one of the two heavy chains.

Therapeutic IgA for cancer treatment has not yet entered clinical trials but there are promising *in vivo* results in mouse models (7–11). It could, therefore, be a valuable addition or alternative for patients that do not respond or have become resistant to IgG therapy. Depending on the location and tumor type, the rigorous activation of neutrophils by IgA could be a very welcome alternative for IgG, particularly since neutrophils

are the most abundant type of leukocyte in the body. Next to this, we and others have demonstrated that simultaneous engagement of FcγRs and FcαRI enhances tumor cell killing (18, 39). Great progress has been made in cancer treatment since the modulation of immune checkpoint molecules was included in immunotherapy (40, 41). This has primarily been focused on eliciting T-cell immunity against the tumor. Likewise, myeloid cells express checkpoint molecules as well. The most prominent example is the myeloid-restricted signal regulatory protein alpha (SIRPα) that transduces the "don't eat me" signal when bound to the ubiquitously expressed CD47 (42). Recent work has demonstrated that blocking SIRPα-CD47 axis potentiates IgG antibody therapies (43), but even more so enhances the therapeutic potential of IgA monoclonals [(44) Treffers et al., submitted].

In conclusion, we have demonstrated that unstimulated primary neutrophils are able to kill IgA-opsonized tumor cells efficiently in contrast to IgG. Our current model (**Figure 5**) suggests that the strong induction of FcαRI signaling is crucial for this process. Taken together, these promising developments support a solid base for exploring the possibilities of IgA therapeutics further and improve future treatment of cancer.

AUTHOR CONTRIBUTIONS

JL, TtB, AB, and TV: conceptualization; TtB, JJ, AB, SB, ME, RK, MN, and JL: methodology; AB, TtB, SB, ME, MN, JJ, and RK: formal analysis; TR: providing crucial reagents; TtB, AB, SB, JJ, and RK: investigation; TtB and JL: writing—original draft; TtB, AB, ME, JJ, MN, RK, SB, JL, and TV: writing—review and editing; AB and TtB: visualization; TtB and JL: supervision; TtB, TV, and JL: funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00704/full#supplementary-material

Figure S1 | Stills from live-cell microscopy of anti-CD20 IgA and anti-EGFR IgA facilitated tumor cell killing by PMNs. **(A)** Calcein labeled EL4-CD20 cells or **(B)** A431 cells were imaged together with unstimulated human PMNs in the presence of anti-CD20 IgA1 **(A)** or anti-EGFR IgA2 **(B)**. Killings of target cells are indicated by the colored circles with corresponding time points above the images. Effector:target ratio for anti-CD20 IgA1 **(A)** was 15:1 and **(B)** for anti-EGFR IgA2 10:1.

Figure S2 | Quantitative expression of FcγR and FcαRI on primary human monocytes **(A)** or NK cells **(B)** of $n = 6$–11 healthy donors using flow cytometry (Qifikit).

Figure S3 | Remaining individual donors from which PMN binding dynamics to anti-CD20 IgG1or IgA opsonized Daudi cells were measured using ligand tracer technology.

Figure S4 | (A,C) All five individual donors for p-ERK induction, showing the p-ERK and ERK blots. **(B)** Quantification of the anti-CD20 blots, by dividing the p-ERK signal over the ERK signal. The 0 min time point was set to 1. A.U. = arbitrary units, 3 donors are combined. Statistics: two-way ANOVA with Tukey's multiple comparison test. Data are mean + SEM (note: all other graphs in paper are mean + SD), $p < 0.05$: $*$.

Video S1 | Anti-HER2 IgA2 (5 μg/ml) mediated killing of calcein labeled adhered A431-HER2 cells by unstimulated primary human neutrophils. Tumor cell lysis is visualized by the red fluorescence of the DNA dye TO-PRO™-3.

Video S2 | Live-cell imaging of adhered A431-HER2 cells in the presence of Anti-HER2 IgG1 (5 μg/ml, trastuzumab), TO-PRO™-3, and unstimulated primary human neutrophils.

Video S3 | EL4-CD20 were labeled with calcein and live-cell imaged in the presence of anti-CD20-IgA1 (5 μg/ml) and unstimulated primary human neutrophils, E:T = 15:1.

Videos S4,5 | Live-cell imaging of calcein labeled A431 cells in suspension together with anti-EGFR IgA2 (5 μg/ml) and unstimulated primary human neutrophils, E:T = 10:1.

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REVIEW
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Understanding Fc Receptor Involvement in Inflammatory Diseases: From Mechanisms to New Therapeutic Tools

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Fc receptors (FcRs) belong to the ITAM-associated receptor family. FcRs control the humoral and innate immunity which are essential for appropriate responses to infections and prevention of chronic inflammation or auto-immune diseases. Following their crosslinking by immune complexes, FcRs play various roles such as modulation of the immune response by released cytokines or of phagocytosis. Here, we review FcR involvement in pathologies leading notably to altered intracellular signaling with functionally relevant consequences to the host, and targeting of Fc receptors as therapeutic approaches. Special emphasis will be given to some FcRs, such as the FcαRI, the FcγRIIA and the FcγRIIIA, which behave like the ancient god Janus depending on the ITAM motif to inhibit or activate immune responses depending on their targeting by monomeric/dimeric immunoglobulins or by immune complexes. This ITAM duality has been recently defined as inhibitory or activating ITAM (ITAMi or ITAMa) which are controlled by Src family kinases. Involvement of various ITAM-bearing FcRs observed during infectious or autoimmune diseases is associated with allelic variants, changes in ligand binding ability responsible for host defense perturbation. During auto-immune diseases such as rheumatoid arthritis, lupus or immune thrombocytopenia, the autoantibodies and immune complexes lead to inflammation through FcR aggregation. We will discuss the role of FcRs in autoimmune diseases, and focus on novel approaches to target FcRs for resolution of antibody-mediated autoimmunity. We will finally also discuss the down-regulation of FcR functionality as a therapeutic approach for autoimmune diseases.

Keywords: immunoglobilins, Fc receptor, antibody treatment, signaling/signaling pathways, inflammatory diseases

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FC RECEPTOR MODES OF ACTION

Immunoglobulin Fc receptors (FcRs) are membrane molecules expressed by several hematopoietic cells that recognize the Fc region of several immunoglobulin (Ig) classes and subclasses. We distinguish FcR for IgG (FcγRI/CD64, FcγRII/CD32, and FcγRIII/CD16), IgE (FcεRI), IgA (FcαRI/CD89), IgM (FcμR), and IgA/IgM (Fcα/μR). Several other receptors expressed on different

cell types also bind Ig molecules: neonatal FcR for IgG (FcRn) on intestinal epithelium, placenta, and endothelium, low affinity FcεR (FcεRII/CD23) on B cells and macrophages, and polymeric Ig receptor (pIgR) on mucosal epithelium (1–3).

The function of antibodies depends on one hand on their ability to recognize antigenic epitopes and, on the other hand, on their dynamic flexibility and their capacity to interact with their cognate FcRs. Engagement of FcRs expressed by leukocytes initiates a number of pro-inflammatory, anti-inflammatory, and immune modulatory functions in the host adaptive immune responses leading to protection but sometimes also to disease.

Several FcRs require the Immunoreceptor Tyrosine-based Activation Motif (ITAM; with the sequence $Yxx[L/I]x_{(6-8)}Yxx[L/I]$) present in the cytoplasmic tail of the receptor or of associated subunits (FcRγ or FcεRIβ chain) to induce cell signaling. ITAM-mediated functions include phagocytosis, degranulation, antibody-dependent cellular cytotoxicity (ADCC), cytokine, lipid mediator and superoxide production, all of which depend on the cell type and on outside-in signals induced by the ligand. Engagement of the type I FcRs by immune complexes, induces receptor aggregation followed by activation and recruitment of Src family kinases (SFKs), such as Lyn and Fyn (4). The former induces the phosphorylation of the conserved tyrosines in the ITAM motif, followed by activation and recruitment of the tyrosine kinase Syk. This process activates various proteins involved in cell response, such as Phospholipase C gamma 1 (PLCγ), Bruton's tyrosine kinase (Btk), guanine nucleotide exchange factor Vav and phosphoinositide 3-kinase (PI3K). Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5) P_2) by PLCγ generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) leading to calcium mobilization and protein kinase C (PKC) activation, respectively. Calcium influx and PKC activation promote cell responses such as degranulation and cytokine production. Vav plays also an important role in actin cytoskeleton remodeling to control phagocytosis and superoxide production by NADPH oxidase. PI3K catalyzes the phosphorylation of $PtdIns(4,5)P_2$ into $PtdIns(3,4,5)P_3$ in the plasma membrane. Pleckstrin homology domains contained in proteins such as PLCγ, GRB2-associated-binding protein 2 (Gab2), protein kinase B (PKB/Akt) and Btk, bind $PtdIns(3,4,5)P_3$ thus recruiting them at the inner leaflet of the plasma membrane promoting their phosphorylation and activation (**Figure 1,** left).

The activation of ITAM-bearing immune receptors can be retro-controlled by ITIM-bearing inhibitory FcRs such as the FcγRIIB. The ITIM motif is defined by a single [I/V/L/S]xYxx[L/V] sequence. However, inhibition of cell activation by this motif requires co-ligation between the inhibitory and heterologous activating receptors by immune complexes promoting the recruitment of inositol phosphatases (SHIP-1 and SHIP-2) (6) (**Figure 1,** Middle). Another inhibitory mechanism has been recently identified that involves ITAM itself. Indeed, following low avidity ligand interactions, ITAM-bearing FcRs induce a sustained inhibitory signal without co-ligation with heterologous receptors. This mechanism was involved in the maintenance of immune homeostasis (7–14). We named this ITAM-mediated inhibitory signal, ITAMi. It has been shown that several low affinity receptors, such as FcαRI, FcγRIIA and FcγRIIIA, can function as such bi-functional receptors to induce either activating or inhibitory signals, a property that can be exploited to reduce the susceptibility to autoimmune and inflammatory diseases (11). Monovalent or divalent targeting of FcRs bearing an ITAM motif induced ITAMi signals that involved activation and recruitment of the Src homology region 2 domain-containing tyrosine phosphatase SHP-1 (**Figure 1,** Right). It has been demonstrated that other immunoreceptors such as the antigen receptors BCR and TCR can also associate with SHP-1 upon interaction with low avidity ligands (15, 16). Moreover, SHP-1 deficiency in hematopoietic cells favors development of various auto-immunes diseases. For example, the motheaten mice (mev/mev) which express approximately 20% wild type activity of SHP-1, develop severe immune dysregulation and autoantibody production (17).

During ITAMi signaling induced by FcRs, Lyn is essential for the phosphorylation (on tyrosine residue 536) and the activation of SHP-1 (4). It has been reported that Lyn is involved in positive and negative signals induced by antigen receptors (18, 19). Lyn plays an important role in the negative selection of B cells in the bone marrow, since the absence of Lyn was associated with a decreased B cell number in the periphery of mice. In the absence of Lyn, other SFKs, such as Fyn, act as positive regulators of BCR signaling, suggesting a loss of anergy. The opposite roles of Lyn and Fyn were recently demonstrated by *in vivo* approaches. Lyn deficiency aggravates auto/inflammatory diseases such as nephritis and arthritis, while the absence of Fyn protects against these diseases (4). Additionally, we showed that activation of leukocytes in lupus nephritis patients was associated with Fyn-activated signature, suggesting that the balance between Lyn and Fyn is dysregulated during diseases.

Another FcR that play an essential role in the transcytosis by epithelial cells of dimeric IgA, but also pentameric IgM (notably during IgA deficiencies), is called the polymeric immunoglobulin receptor (pIgR). The pIgR is internalized with its ligands by endocytosis and transcytosed from the basolateral membrane into apical side of the epithelial cell (20). The central role of this receptor is to generate secretory IgA (formed of IgA dimers linked to the extracellular domain of the pIgR, also known as secretory component) in exocrine secretions to establish host-microbiota symbiosis and to mediate the protection of mucosal surfaces against pathogens (20, 21). The Fcα/μR, the Fc receptor for IgA and IgM, may play a role in systemic and mucosal immunity. It has been shown that none of the B cells, T cells, monocyte/macrophages, or NK cells in human blood samples expressed this receptor irrespective of age, ethnic origin or gender. Its expression is restricted to B cells from germinal center, follicular dendritic cells and tonsillar cells. Although, the exact function of the Fcα/μR is not fully clarified, it may play an important role in antigen presentation and B cell selection in the germinal center responses (22).

FcRs are divided into type I and type II on the basis of the conformational state of the Ig Fc domain that interacts with the receptor (1, 23). Type I Fc receptors interact with "open," but not "closed" Ig Fc conformation (**Figure 2**). These receptors include

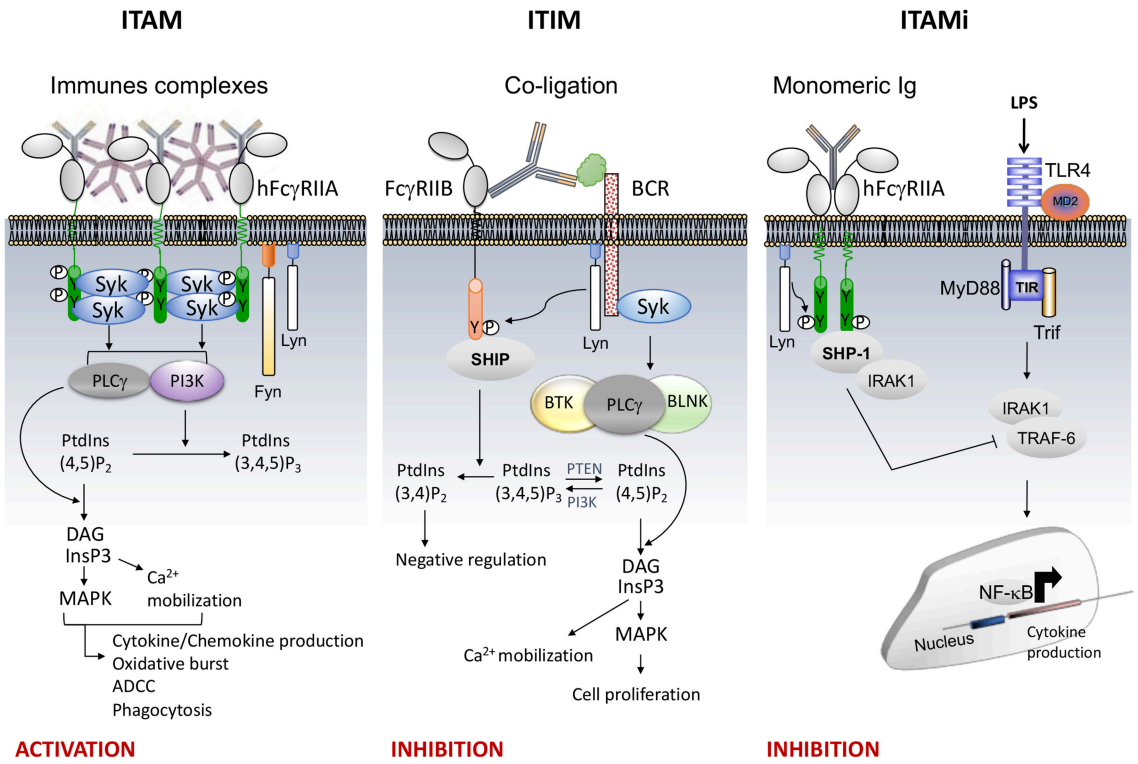

FIGURE 1 | FcR signaling (e.g., FcγRII). **(Left)**, the aggregation by an immune complex of FcR bearing ITAM motif (e.g. FcγRIIA) induces phosphorylation of the two ITAM tyrosine residues by Src kinases Lyn and Fyn responsible for recruitment and phosphorylation of Syk inducing cellular activation through PLCγ and PI3K signaling pathways. The PLCγ converts $PI(4,5)P_2$ into IP_3 and DAG. IP_3, a soluble inositol phosphate, leads to Ca^{2+} mobilization while DAG activate MAPK.PI3K converts $PI(4,5)P_2$ to $PI(3,4,5)P_3$ allowing recruitment of signal intermediates through their pleckstrin homology (PH) domain **(Middle)**, co-ligation between an activating heterologous receptor (e.g., the BCR) and the inhibitory FcR (i.e., FcγRIIB) induces phosphorylation of the tyrosine present within the ITIM motif by Lyn (5), leading to the phosphorylation and recruitment of phosphatases (SHIP or SHP). The phosphatases PTEN and SHIP1/2 regulate cellular levels of $PI(3,4,5)P_3$ by hydrolyzing it to $PI(4,5)P_2$ and $PI(3,4)P_2$, respectively. These dephosphorylations inhibit cell proliferation. **(Right)**, monovalent targeting of FcR bearing ITAM motif (e.g., FcγRIIA) induces the phosphorylation of the last tyrosine residue of the ITAM motif by Lyn responsible for transient recruitment of Syk followed by that of SHP-1 which abrogates the activation signal.

FcγRI, FcγRII, FcγRIII, FcεRI, FcαRI, FcμR, and Fcα/μR (25–29). In contrast, type II FcRs, bind preferentially Ig Fc domains in "closed" conformation. Among these are C-type lectin receptors such as FcεRII (CD23) and DC-SIGN (**Figure 3**).

For type II Fc receptors, glycosylation of the Fc domain induces a conformational change that occludes the binding site for type I Fc receptors that lies near the hinge region (open conformation) and reveals a binding site at the CH2-CH3 domain interface (closed conformation). These receptors bind antibodies in a two receptors-to-one antibody stoichiometry that may influence signal initiation (1). DC-SIGN and SIGN-R1, for example, bind secretory IgA and play an intriguing role in dendritic cells inducing IL10 and Treg-mediated tolerance (30). Signaling through these receptors, however, is not yet documented as compared to type I FcRs, with the exception of CD23. Crosslinking of CD23 on B cells activates cAMP (31) and intracellular calcium flux (32) which is associated with the activation of the SFK Fyn and of the PI3K pathway (33). These findings are in agreement with our recent data on type I FcRs (4), and indicates that Fyn also plays an activating role in B cells through type II Fc receptors.

FC RECEPTORS AND DISEASES

Gene Alleles

Several single-nucleotide polymorphisms (SNPs) have been reported in the genes encoding activating FcγRs (FcγRIIA, FcγRIIIA, and FcγRIIIB). In the gene encoding the inhibitory FcγRIIB, a SNP has been described which is associated with autoimmune diseases such as SLE and rheumatoid arthritis (RA) (34, 35). In addition to SNPs, copy-number variations (CNVs) of FcγR genes are associated with susceptibility to autoimmune disorders (34–40). Most polymorphisms concern the extracellular domains which bind to IgG, affecting the affinity between these receptors and IgG subclasses. However, no polymorphism and CNV have been clearly identified for FcγRI.

The most studied polymorphism is the one in the second Ig-like extracellular domain of the FcγRIIA that results in a point mutation of amino acid at position 131, coding for either arginine (R131) or histidine (H131). FcγRIIA-R131 binds less efficiently IgG2 than FcγRIIA-H131 (34). This *Fcgr2a* polymorphism has been described as a heritable risk factor for autoimmune and infectious diseases (41, 42). Moreover, genome-wide

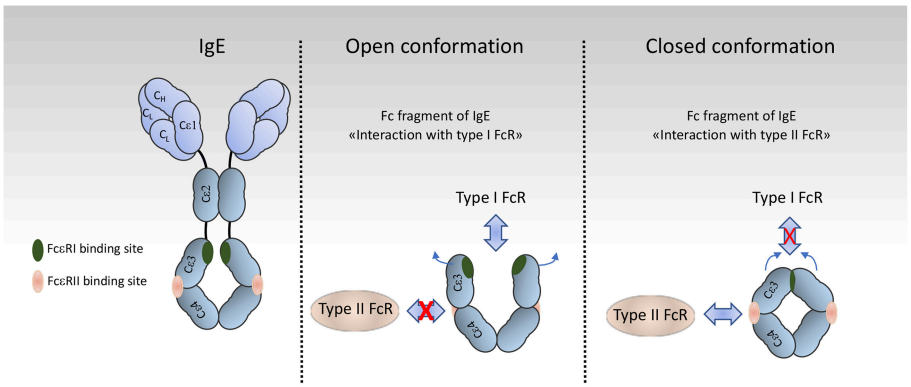

FIGURE 2 | Organization and conformational rearrangements of the IgE Fc. **(left)**, IgE and the binding sites to FcεRI (green) and to CD23 (pink) [adapted from Pennington et al. (24)] **(Middle** and **Right)**. Representation of the open and closed conformations, respectively, of the IgE Fc Cε3–4 domains, and the mutual allosteric inhibition by FcεRIα (green) and CD23 (pink).

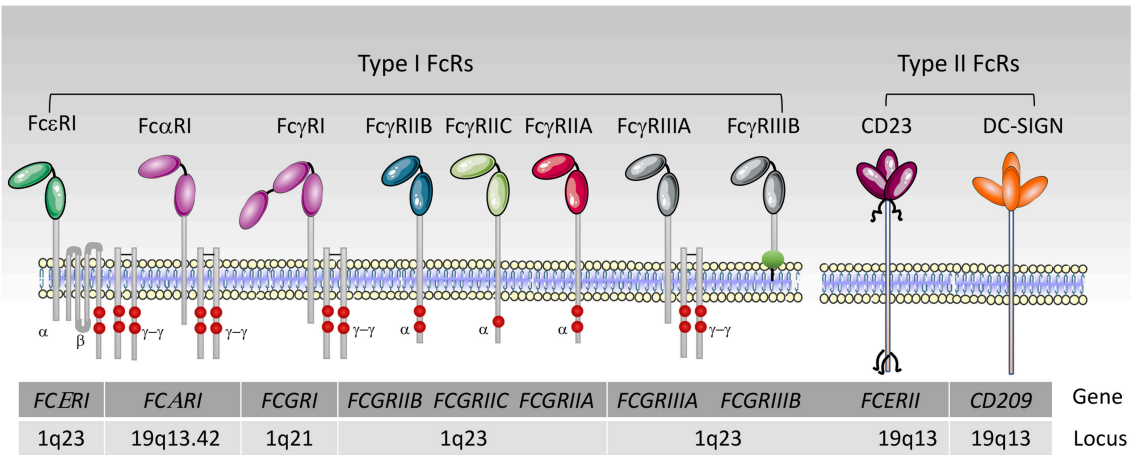

FIGURE 3 | Human type I and II Fc receptors**.** Schematic representation of human Fc receptors at the cell membrane and their association or not with the FcRγ-chain dimer and the FcεRIβ chain, red circles represent the tyrosine residues. The FcγRIIIB is anchored into the outer leaflet of the plasma membrane by a phosphatidylinositol-glycan (green circle).

association studies (GWAS) revealed that FcγRIIA-H131 variant is associated with higher susceptibilities to develop Kawasaki disease and ulcerative colitis (43, 44). This variant is also associated with Guillain–Barré syndrome (45), supporting that immune complexes that include IgG2 auto-antibodies are involved in inflammatory responses. In contrast, the genotype homozygous for the FcγRIIA-R131 variant-encoding gene is associated with SLE, immune thrombocytopenic purpura (ITP) and IgA nephropathy (IgAN), revealing a complex and contrasted picture for the role of IgG2-containing immune complexes in autoimmune diseases (46–48). Regarding infectious diseases, neutrophils homozygous for the gene encoding the H131 variant show a higher capacity for IgG2-mediated phagocytosis than those homozygous for the gene encoding the R131 variant (41). In agreement, patients with the R encoding allele were found to have more severe cases of Severe Acute Respiratory Syndrome infection and were more susceptible to encapsulated microorganism infection, which was attributed to poor IgG2 binding to the R131 variant of FcγRIIA (41, 42). As mice do not express FcγRIIA homologs, transgenic mice expressing the *Fcgr2a* human gene encoding the R131 variant develop spontaneously autoimmune diseases such as pneumonitis, glomerulonephritis and RA (49, 50). Moreover, the fact that FcγRIIA-R131 expressed on the FcRγ-/- background in mice similarly develop thrombocytopenia (51) and arthritis (11, 52) indicates that pro-autoimmune signals through FcγRIIA-R131 ITAM were sufficient to induce diseases. Biochemical analyses showed that two tyrosines of FcγRIIA ITAM motif were needed to induce inflammatory signals (53). Taken together, these animal models underline the critical involvement of *Fcgr2a* polymorphism in a number of diseases.

A polymorphism has been found in the inhibitory FcγRIIB-encoding gene that leads to a single I-to-T amino acid substitution in the transmembrane domain (residue 232)

(43, 54). Human monocytes expressing the FcγRIIB-T232 failed to inhibit heterologous receptors-mediated cell activation (55). The FcγRIIB-T232 polymorphism is associated with susceptibility to develop auto-immune diseases such as SLE (42, 54, 56). However, there are some discordances concerning the SNPs in the promoter region of *Fcgr2b*, such as −386G and −120T (haplotype 2B.2), and −386C and −120A (haplotype 2B.4) variants. It has been shown that the 2B.4 SNP promoter haplotype upregulates the expression of FcγRIIB on neutrophils and monocytes that negatively correlates with lupus nephritis (46). This is in agreement with previous reports in mouse SLE-like models and suggests that FcγRIIB expression is protective in SLE (57). However, in striking contrast and in an apparent paradox, the same 2B.4 promoter haplotype was found by the same authors to correlate with SLE (46). This positive (SLE) vs. negative (lupus nephritis) paradoxical association of the 2B.4 promoter haplotype suggests multifaceted impacts of FcγRIIB in SLE that may depend on the affected cell types (e.g., monocytes *vs.* neutrophils). Alternatively, particular cell types expressing FcγRIIB could have aggravating or protective actions in SLE depending on which affected tissue these cells are recruited to, or on how these cells impact the systemic *vs*. local aspect of the disease. Thus, further investigation is necessary to elucidate the association of the promoter haplotype in disease development.

The *Fcgr3a* polymorphism is characterized by a point mutation in the codon for residue 158, encoding valine (V158) or phenylalanine (F158) in the Ig-like domain near the membrane (34–36). The FcγRIIIA-V158 variant has a higher affinity for all human IgGs than the FcγRIIIA-F158 variant (40). The FcγRIIIA-F158 is associated with susceptibility to SLE, Crohn's disease and Behçet's disease (35, 36). Although studies have also explored the association between RA and the V or F 158 variant, their results have been contradictory and this question remains unsettled (58, 59).

Several *Fcar1* polymorphisms have been found, including two in the functional promoter region of the FcαRI encoding gene (−114T/C and +56T/C relative to the major transcription start site) (60). The incidence of the −114C/C polymorphism in patients with IgAN was significantly increased compared with other chronic kidney diseases (CKD) and healthy donors (HD) (15.6 vs. 4.0% in other CKD and vs. 2.4% in HD). This *Fcar1* polymorphism in the promoter region appears to be associated with susceptibility to IgAN, suggesting the importance of FcαRI expression in this disease. A third *Fcar1* polymorphism has been described in the coding region for FcαRI, which changes codon 248 from AGC to GGC leading to G248 instead of S248 in the cytoplasmic domain of the receptor (61). Interestingly, these two different alleles demonstrate significantly different FcαRI-mediated intracellular activating signaling. The proinflammatory FcαRI-G248 variant has been associated with SLE in two ethnic groups (61). However, this *Fcar1* polymorphism was not associated with other auto-immune diseases such as systemic sclerosis, RA or IgAN (62, 63). A fourth *Fcar1* polymorphism (A/G at nt 324) was also associated with aggressive periodontitis (64). Patients displaying the nt 324 A/A allele presented polymorphonuclear neutrophil dysfunctions with a decreased phagocytosis of periodontopathic bacteria (Porphyromonas gingivalis) as compared to patients expressing the nt 324 G/G (64).

Regarding the pIgR, it has been reported as a susceptibility gene for nasopharyngeal cancer (NPC) associated with Epstein-Barr virus (EBV) (65). This lead to a hypothesis that pIgR could be the nasopharyngeal epithelial receptor for EBV via IgA-EBV complex. Transcytosis failure due to missense C → T mutation on the *PIGR*1739 nucleotide (resulting in an A-to-V mutation near the endoproteolytic cleavage site of pIgR) could decrease the ability of pIgR to release IgA-EBV complexes, thus increasing susceptibility to develop NPC (65).

The high-affinity FcεRI is expressed by mast cells and basophils and plays an important role in allergic diseases. Several studies have identified two FcεRI polymorphisms associated with allergies. The −66T > C and/or the −315C > T are associated with atopic dermatitis, chronic urticaria, asthma, and high serum IgE levels (66–69). These polymorphisms were also associated with allergic inflammatory diseases such as atopy and nasal allergy (70, 71).

Table 1 summarizes most of described FcR alleles and their expression and functions in physiology and pathology (42–44, 46, 48, 60, 65, 66, 69, 72–83).

Alterations in FcR Expression

Several studies highlighted altered expression, structure and function of FcγRs in patients. Whereas, $CD4^+$ T cells from healthy donors fail to express significant levels of FcγR, FcγRIIA is expressed in a subpopulation of $CD4^+$ T cells in blood samples from HIV-1-positive patients and is highly enriched in inducible replication-competent proviruses suggestive of an $Fc\gamma RIIA^+$ HIV reservoir (84). Yet, in other studies, FcγRIIA expression did not selectively enrich for HIV- or SIV-infected $CD4^+$ T cells in peripheral blood or lymphoid tissue since resting $Fc\gamma RIIA^+$ $CD4^+$ T cells have <3% of the total HIV DNA amongst $CD4^+$ T cells (85, 86). Taken together, whereas FcγRIIA expression in $CD4^+$ T cells becomes a marker for HIV infection, the involvement of $Fc\gamma RIIA^+$ $CD4^+$ T cells in AIDS remains to be elucidated.

The inhibitory FcγRIIB, in contrast, is down-regulated in autoimmune diseases notably on both memory and plasma B lymphocytes of active SLE patients compared to those from healthy individuals (87). However, this down-regulation was not seen on myeloid-lineage cells. This was also observed in Hashimoto's thyroiditis (88).

High level of FcγRIIIA expression on monocytes together with that of CD14 is associated with proinflammatory cytokine profiles and higher potency in antigen presentation allowing to define monocyte subsets with distinct phenotypes and functions (89).

For FcαRI, its expression is dysregulated in patients with AIDS, ankylosing spondylitis, alcoholic liver cirrhosis, Henoch-Schonlein purpura (HSP) and IgAN (90–93). Some of these studies have shown biochemical abnormalities revealing FcαRI altered protein mobility in SDS-PAGE suggesting altered glycosylation of this receptor (90). Interestingly, mutational studies of FcαRI indicate that the N58 residue of the receptor controls IgA-binding enhancement (94). In parallel,

TABLE 1 | Human FcRs: their expression, function and allotypes.

Name	Subclass binding	Expression	Functions	Alleles	Link to diseases
FcγRI (CD64)	IgG 1/3/4	Monocytes/Macrophages Neutrophils/DCs/Mast cells	Activation	–	–
FcγRIIA (CD32a)	H_{131}:IgG 1/2/3/4 R_{131}:IgG I/(2) /3/4	Monocytes/Macrophages Neutrophils/DCs/Basophils/Mast eells/Eosinophils	Activation/inhibition	H131/R131	Kawasaki diseases (43), Ulcerative colitis (44). Childhood-onset ITP (71)/Lupus (46), IgAN (48), arthritis
FcγRIIB (CD32b)	IgG I/(2)/3/4	B cells/DCs/Mast cells/Basophils	Inhibition	Promoter−3S6C or −120/T232	Lupus (46)/lupus (42), Atopy (72)
FcγRIIC (CD32c)	IgG 1/(2)/3/4	NK cells/ Monocytes/Macrophages /Neutrophils	Activation	Q13/stopI3	Kawasaki disease (73)
FcγRIIIA (CD16)	V_{158}: IgG I(2)/3/4 F_{I58}: IgG I/(2)/3/4	NK cells/ Monocytes/Macrophages	Activation/Inhibition	V15S/F158	IgAN (48), arthritis seventy (74), childhood chronic ITP (75)/Lupus (76), arthritis (58), Crohn's disease (77)
FcγRIIIB (CD16b)	IgG 1/3/4?	Neutrophils/ Eosinophils/ Basophils	Activation		Wegener's granulomatosis (78)
FcαRI (CD89)	IgAI, IgA2, CRP	Monocytes/Macrophages Neutrophils/DCs/Kupffer cells (79)	Activation/Inhibition	114T/C	IgA nephropathy (60), AIDS, ankylosing spondylitis, alcoholic liver cirrhosis, Henoth-Schonlem purpura (HSP) (26)
FcμR	IgM	B and T lymphocytes	Inhibition/?		Chronic Lymphocytic Leukemia (CLL) (80, 81)
Fcα/μR	IgM and IgA	Germinal center B cell, Follicular dendritic cells	?		
FcεRI	IgE	Mast cells/Basophils	Activation	66T/31SC	Atopic dermatitis.$_J$ asthma and chronic urticaria (66–71)
FcεRII (CD23)	IgE	B cells and macrophages	Activation	–	AIDS and B-CLL (82)
FcRn	IgG 1/2/3/4	Monocytes/Macrophages Neutrophils/DCs/endothelium/ Syncytiotrophoblasts	Recycling Transport uptake	VNTR1–5	
PIgR	pIgA	Mucosal epithelium	Transcytosis	1739C to T	Nasopharyngeal cancer and infection (65)

From left to right columns: names, function, alleles that include amino acid variations in immunoglobulin domains and the transmembrane domain, cellular expression of FcRs, and diseases linked to alleles and CNVs (reference numbers are shown), and binding abilities of IgG subclasses to each FcR allele. IgAN, IgA nephropathy; CLL, chronic lymphocytic Leukemia; FcRn, neonatal Fc receptor; AIDS, acquired immune deficiency syndrome; PIgR, Polymeric immunoglobulin receptor; DCs, dendritic cells.

abnormally glycosylated IgA1 molecules (hypogalactosylation and hyposialylation on the hinge region) observed in patients with IgAN and HSP is associated with the shedding of a soluble form of FcαRI (sFcαRI), which participates in the formation of circulating IgA1 complexes (95). These IgA1-sFcαRI complexes were decreased in serum of IgAN patients with severe and progressive disease as compared to non-progressive IgAN patients (96) suggesting a kidney deposition, and hence a possible nephrotoxic action, of such complexes which is further supported by studies in IgAN patients with recurrence of the disease after kidney transplantation (97). In this study, IgA-sFcαRI complexes were decreased in the serum of patients with recurrent IgAN and sFcαRI was detected in the kidney mesangium only in patients with the recurrent disease. Direct evidence for a nephrotoxic role of IgA1-sFcαRI complexes were obtained in humanized animal models. These experimental studies were based on the fact that mouse do not have homologs of IgA1 and FcαRI. Humanized mice expressing human IgA1 and human FcαRI spontaneously develop mesangial deposits of IgA1-sFcαRI complexes (98). In the glomeruli, these complexes are captured by the transferrin receptor 1 (TfR1), which is upregulated on mesangial cells, through interaction with polymeric (p) IgA1 and FcαRI (98, 99). Although the mechanism of TfR1 upregulation remains poorly understood, the crosslinking enzyme transglutaminase 2 has been found to be overexpressed and associated with the receptor controlling mesangial IgA1 complex deposition and renal injury (98). Polymeric IgA1 induce TfR1 expression *in vitro* on mesangial cells. Polymeric IgA1-TfR1 interaction triggers activating signals through mTOR, PI3K and ERK pathways, and phosphorylated ERK is associated with disease progression (100). Interestingly enough, in physiology TfR1-IgA1 interaction plays a role in erythropoiesis (101). Progression of IgAN to end-stage renal disease may also involve FcαRI activation on tissue macrophages surrounding hypogalactosylated IgA1-mediated mesangial lesions. Indeed, $FcαRI_{R209L}$Tg mice, with an R-to-L substitution at position 209 in the transmembrane region of FcαRI, did not develop macrophage infiltration and proteinuria (102). This mutant receptor cannot associate with the ITAM-bearing FcRγ signaling subunit (103). In agreement, only macrophages expressing wild-type FcαRI, but not those expressing $FcαRI_{R209L}$, were able to migrate to the kidney after adoptive transfer demonstrating that their chemotaxis depends

on the FcRγ subunit. Of note, FcαRI can be found associated and non-associated with FcRγ on the same cells (95). Since mouse IgA and human FcαRI interaction may be sufficient to induce receptor shedding leading to IgA deposits in the kidney, we hypothesized that both receptor types could cooperate to induce disease, the FcRγ-less FcαRI allowing IgA deposits and the FcRγ-associated FcαRI promoting inflammatory cell infiltration and disease progression (102).

For FcμR (IgM receptor), deficiency in the receptor in mice revealed that this receptor plays a crucial role in B cell responses (27, 104). Mice deficient for *Fcmr* are characterized by the increase in pre-immune serum IgM, dysregulation of humoral immune responses, disturbances in B cell subpopulations, B cell proliferation alteration after BCR ligation, and autoantibody production (104, 105). Accordingly, in chronic lymphocytic leukemia (CLL), the membrane expression and the soluble form of FcμR in serum were increased. The potential mechanism proposed for the up-regulation of FcμR is that the antigen-independent self-ligation of BCR on CLL cells induces activation of Syk thus increasing the cell surface expression of FcμR. Furthermore, the IgM antibodies produced by CLL cells that had differentiated into plasma cells, recognized soluble or lymphocyte membrane self-antigens. IgM/self-antigen immune complexes would then crosslink FcμR and BCR favoring cell survival. An alternative splice variant of the soluble FcμR is increased in CLL patients, but its biological function is unclear (105, 106).

For type II FcRs, an increased expression of FcεRII on monocytes in AIDS patients has been associated with the aberrant activated phenotype of these cells during the immunopathogenesis of AIDS. Interestingly, despite the known ability of IL-10 to downregulate monocyte FcεRII expression, in AIDS the IL-10-enriched environment is not associated with the suppression of FcεRII expression on monocytes (82) indicative of an impairment of this negative regulation in patients. In B-CLL also, patients strongly express FcεRII, which is associated with B cell activation and proliferation. Moreover, altered phosphorylation of FcεRII intracellular tail were reported in B-CLL B lymphocytes (107) further supporting an active role of FcεRII in this disease.

TARGETING OF FC RECEPTORS AS THERAPEUTIC APPROACHES

Blocking/neutralizing Activating Receptor Antibodies

Both murine models and studies in patients suggest a major role of the activating FcRs in initiating and propelling immune complex-mediated inflammatory reactions. For example, human FcγRIIA transgenic mice are hypersensitive to pathogenic antibodies and develop destructive arthritic syndromes. *Ex vivo* experimentation with circulating monocytes from RA patients suggest that FcγRIIA is responsible for the production of reactive oxygen species (11, 108). Anti-receptor monoclonal antibodies, intact antibodies and antibody fragments as well as a variety of small molecules have been designed to interact with the Ig-binding domains in activating FcRs. Some of these approaches have shown encouraging results when tested *in vitro* or *in vivo* for blocking immune complex-mediated cell effects and inflammation. Recently, we have demonstrated that divalent targeting of FcγRIIA by anti-hFcγRII F(ab)′2 fragments ameliorates RA-associated inflammation. This therapeutic effect was mediated by the induction of inhibitory ITAM (ITAMi) signaling through the activation of SHP-1. Moreover, treatment of inflammatory synovial cells from RA patients by F(ab′)2 fragment of hFcγRIIA-specific antibody inhibited production of reactive oxygen species associated with the induction of FcγRIIA-mediated ITAMi signaling. These data suggest that targeting of hFcγRIIA by specific antibody such as clone IV.3 mAb could ameliorate RA-associated inflammation (11). Anti-FcαRI Fab and F(ab)′2 fragments also have demonstrated efficiency on RA (109). Interestingly, in autoimmune blistering skin diseases that involve interaction between IgA autoantibodies and the neutrophil FcαRI, targeting FcαRI by blocking peptides or antibodies prevents neutrophil migration and tissue damage *ex-vivo* (110, 111).

In allergy, treatment by anti-IgE antibodies has been considered a therapeutic option for a long time. The recombinant anti-IgE humanized monoclonal antibody-E25, named "omalizumab," is now used in several clinical trials and shows efficacy against IgE-mediated allergic reactions (112, 113) through inhibition of IgE binding to FcεRI on the surface of mast cells and basophils (113).

The above-described upregulation of FcμR expression in CLL cells is of significant clinical interest. It can be easily evaluated by flow cytometry on cells and, additionally, the levels of soluble FcμR may correlate with disease progression. Thus, it may be used as a new biomarker for CLL (106). FcμR is a good target also because it is involved in the pathogenesis of CLL and in the progression of the disease through support of leukemic cell survival (80). Hence, disrupting CLL survival signals might be achieved through FcμR therapeutic targeting. However, a large cohort of CLL patients will be required to validate these two applications (106).

IVIG

Intravenous immunoglobulins (IVIG) are harvested from the pooled plasma of 3,000 to 100,000 healthy donors. They consist of over 95% IgGs with a subclass distribution corresponding to that found in normal human serum (114). IVIG is used in treatment of several immunodeficiency diseases including idiopathic thrombocytopenic purpura (ITP), Kawasaki disease, and neurologic diseases such as Guillain–Barre syndrome, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, sclerosis, and autoimmune encephalitis (115). In ITP patients, administration of IVIG can efficiently attenuate platelet clearance from the circulation. The first proposed mechanism was the competitive blockage of the activating FcγRs on myeloid cells by IVIG, which in turn decreases autoantibody-mediated platelet phagocytosis and ADCC against platelets (116). Furthermore, in pediatric ITP patients, intravenous administration of the Fcγ fragments prepared from IVIG resulted in a rapid recovery in platelet counts (117) further indicating the role of FcγRs in IVIG action. Another IVIG anti-inflammatory mechanism involves saturation of FcRn, the IgG recycling receptor (118). FcRn plays an important role in the maintenance

of IgG half-life. Therefore, inhibition of autoantibody activity can be induced by the alteration of their interaction with FcRn, impairing their half-life and accelerating their clearing from the circulation. IVIG by competing with autoantibodies for FcRn binding could therefore facilitate their clearing.

A role for the inhibitory FcγRIIB has been proposed to be exclusive in IVIG action to explain their Fc dependent effect (118). This statement was based notably on studies showing a decreased anti-inflammatory effect of IVIG in FcγRIIB-deficient animals. In other studies, a role for FcγRIII in IVIG-mediated inhibition has been reported (119) although the mechanism of action was not clearly established. Recently, we reported that IVIG can control inflammatory responses by ITAMi signaling through FcγRIIA and FcγRIII (10, 11). These data are based on the *in vitro* targeting of FcγRIIA and FcγRIII by IVIG at the physiological concentration of IgG showing an inhibitory effect on endocytosis. This was confirmed by targeting FcγRIIA or FcγRIII with F(ab′)2 fragments of specific antibodies. These results were further supported *in vivo* in mice by targeting these receptors with IVIG or with specific antibodies and this inhibitory effect was abolished in receptor-deficient mice (10, 11). Therefore, IVIG could use a combination of non-exclusive mechanisms to promote protection against auto-immune diseases. Although IVIG is well tolerated, some patients develop immediate or delayed adverse effects depending on the time occurrence. The Flu-like symptoms such as fever, fatigue and nausea are the most frequent adverse effects. For the delayed adverse effects, the most frequent are thrombotic events, neurological disorders and renal failure. These delayed adverse effects are rare but dangerous (120). The majority of adverse effects are associated with high doses of immunoglobulins; thus, determining individual dosages to guarantee the efficacy of therapy and minimize adverse effects is an urgent goal.

Treatment with highly purified serum monomeric IgA (mIgA) decreases cell activation through FcαRI-FcRγ-mediated ITAMi signaling (109). Human mIgA or anti-FcαRI Fab fragments were used to prevent or treat collagen antibody-induced arthritis in FcαRI-transgenic mice. mIgA treatment decreased significantly leukocyte infiltration to the inflamed joints of mice, which was associated with SHP-1 phosphorylation at Y536 residue in joint tissue cells. Moreover, mIgA reversed the activating ITAM to ITAMi signature and the state of inflammation in the synovial fluid isolated from RA patients (109). Of note, protection was also achieved with human serum IgA (4). These findings open new avenues to develop the concept of IVIgA as a new treatment option for inflammatory and auto-immune diseases.

Engagement of the Inhibitory FcγRIIB (Agonist)

The only FcR containing an inhibitory ITIM motif, FcγRIIB, serves as a critical negative regulator in immune complex driven reactions. In mice lacking FcγRIIB auto-immune symptoms are exacerbated, and a partial restoration of FcγRIIB expression in B cells rescued mice from developing an SLE-like phenotype (57, 121). Several FcγRIIB specific mAbs have now been developed (122, 123), one of which, mAb2B6, has been chimerized and humanized to direct myeloid-cytotoxicity against B cells (123). These antibodies have the potential to serve as novel immune suppressors in auto-immunity either by blocking B cell activation or by targeting their destruction. In addition, they may have an advantage over CD20 antibodies for their ability to target plasma cells (124).

Targeting FcRn

Blocking FcRn-IgG interaction to decrease circulating IgG levels is one strategy to treat auto-immune disease (118). In the absence of interaction with FcRn, IgG would be degraded in lysosomes more quickly instead of being recycled back into circulation. One straightforward method would be to use recombinant soluble human FcRn to compete with membrane FcRn for IgG. Another approach to block IgG-FcRn binding would be through engineered "bait" IgG which occupy FcRn thus preventing binding of endogenous IgG. Such "bait" antibodies have been generated with a much higher affinity for FcRn at both acidic and neutral pH, thereby providing effective occupancy of FcRn, competing with, and resulting in, degradation of endogenous IgG. These antibodies are also called "Abdegs": antibodies that enhance IgG degradation (125).

An FcRn-specific blocking mAb would also provide interference with FcRn-IgG interaction. One such mAb, 1G3, was examined in rat passive and active models of myasthenia gravis, a prototypical antibody-mediated auto-immune disease (126). Treatment by 1G3 mAb resulted in amelioration of disease symptoms in a dose-dependent manner together with greatly reduced levels of pathogenic antibody in the serum.

Other Future Strategies to Target FcR-Effectors to Treat Auto-Immune/Inflammatory Diseases

Targeting of FcRs by monomeric immunoglobulins or by F(ab′)2 fragments of specific antibodies, induces ITAMi signaling which involved the recruitment of Lyn, but not Fyn. The Src kinase Lyn, leads to partial phosphorylation of the ITAM motif on tyrosine residues (11), and to the conformational change of SHP-1 that allows its recruitment through its SH2 domains to ITAM phosphotyrosine residues (127). This recruitment induces a Lyn-dependent phosphorylation of SHP-1 on Y536 and to SHP-1 phosphatase activity that inhibits the recruitment of various proteins induced by heterologous receptors (52). In contrast, multivalent crosslinking of immunoreceptors by immune complexes induces the recruitment of both Src kinases Lyn and Fyn to the receptor leading to full phosphorylation of the tyrosine residues present in the ITAM motif. This leads to the activation and the recruitment of the kinase Syk. In parallel, Fyn initiates a signaling pathway involving a PI3K-PKCα axis leading the inactivation of SHP-1 through the phosphorylation of its S591 residue barring its recruitment to the plasma membrane (128). Since S591 phosphorylation on SHP-1 keeps the phosphatase in a closed conformation (127), our recent study showed that the phosphorylation of SHP-1 on S591 residue by Fyn axis renders the Y536 residue inaccessible to Lyn. In agreement, the absence of Fyn favors the phosphorylation of SHP-1 on Y536 by Lyn, despite the crosslinking of FcγRIIA (4). These results suggest that the selective absence or inhibition

of Fyn may abolish inflammation during auto-immune and proinflammatory diseases. Taken together, inhibition of Fyn or of the molecules which are upstream or downstream this SFK reverses inflammation during auto-immune and inflammatory diseases and thus, could be a new therapeutic strategy to decrease the activating ITAM signaling in these diseases. Along these lines, inhibition of PI3K (a major player of the Fyn-PI3K-PKCα axis (4)) prevents RA and lupus nephritis progression in mouse models (129). However, it should be mentioned that since Fyn is essential for activating ITAM signals (i.e., phagocytosis), the inhibition of this SFK may favor infections. Moreover, Fyn plays also other roles independently of FcRs. It has been shown that the absence of Fyn impaired multipolar-bipolar transition of newly generated neurons and neurite formation during the early phase of migration. Additionally, inhibition of Fyn decreased the branching number of the migrating cortical neurons (130). Another important hurdle is that Lyn and Fyn present a high homology and there are currently no selective inhibitory drugs. Therefore, Fyn does not appear as the best target to treat auto-immune and pro-inflammatory diseases. Identification of new targets which are downstream of Fyn and which are expressed specifically by immune cells involved in auto-immune diseases will permit development of new therapeutic strategies for auto-immune diseases that involve FcR.

CONCLUSION

Fc receptors may be responsible for diseases when dysregulated in spite of their physiologic protective function. Unraveling all aspects (expression, function, regulation) of FcR biology should help to define approaches to correct the first and to wield the second to restore homeostasis thus representing new hopes for innovative anti-inflammatory strategies. Progress in these two aspects is currently well underway, already proposing new potent therapeutic tools. The future in this field is a promise of scientific excitement.

AUTHOR CONTRIBUTIONS

SB and RM wrote this review. MB has critically read the manuscript.

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REVIEW
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IgG and Fcγ Receptors in Intestinal Immunity and Inflammation

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Fcγ receptors (FcγR) are cell surface glycoproteins that mediate cellular effector functions of immunoglobulin G (IgG) antibodies. Genetic variation in FcγR genes can influence susceptibility to a variety of antibody-mediated autoimmune and inflammatory disorders, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). More recently, however, genetic studies have implicated altered FcγR signaling in the pathogenesis of inflammatory bowel disease (IBD), a condition classically associated with dysregulated innate and T cell immunity. Specifically, a variant of the activating receptor, FcγRIIA, with low affinity for IgG, confers protection against the development of ulcerative colitis, a subset of IBD, leading to a re-evaluation of the role of IgG and FcγRs in gastrointestinal tract immunity, an organ system traditionally associated with IgA. In this review, we summarize our current understanding of IgG and FcγR function at this unique host-environment interface, from the pathogenesis of colitis and defense against enteropathogens, its contribution to maternal-fetal cross-talk and susceptibility to cancer. Finally, we discuss the therapeutic implications of this information, both in terms of how FcγR signaling pathways may be targeted for the treatment of IBD and how FcγR engagement may influence the efficacy of therapeutic monoclonal antibodies in IBD.

Keywords: Fcγ receptor, IgG, inflammatory bowel disease, intestinal immunity, mucosal infections, neonatal immunity

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INTRODUCTION

The gastrointestinal (GI) tract comprises a series of organs whose primary functions are digestion, absorption, excretion, and to host a vast and diverse community of microbial commensals. The stomach and small intestine contribute to physical and chemical digestion and absorption, while the colon is primarily involved in the desiccation and compaction of waste (1). To aid these functions, the human GI tract is colonized by trillions of microorganisms that together form the microbiome, including at least 1,000 species of bacteria, the major component of the commensal flora. Microbial colonization increases progressively along the GI tract, with the colon harboring 10^{10}-10^{12} organisms per gram of luminal contents and elevated species diversity. In the lower GI tract of healthy individuals, anaerobes dominate, including *Bacteroides*, bifidobacterial, fusobacteria, and peptostreptococci, while aerobes, such as enterobacteria, are present at lower densities (2).

A state of mutualism exists between the host and the commensal microbiota, whereby bacteria benefit from the energy-rich sources of food, and the host salvages essential compounds from indigestible nutrients, such as dietary polysaccharides. In this scenario, the host immune system has an essential role in maintaining on-going symbiosis by limiting tissue invasion by resident microbes and keeping detrimental inflammatory responses at bay. This is achieved by dynamic cross-talk between microbes, intestinal epithelial cells (IECs), and tissue-resident leukocytes (3, 4) and includes the production of anti-microbial peptides and mucus by IECs, the induction and secretion of immunoglobulin (Ig)A by intestinal plasma cells, and the dominance of an anti-inflammatory milieu that suppresses damaging responses to luminal microbes. In return, the microbiota interacts with and educates the intestinal immune system, with consequences for both local and system inflammation.

While classically associated with systemic pro-inflammatory responses, recent studies have demonstrated that constitutive production of GI anti-microbial IgG is a feature of adult homeostasis with roles in immune cell maturation, defense against infection, and maternally-acquired neonatal immunity (5–8). Furthermore, historical observations of *de novo* anti-microbial and autoreactive IgG in patients with inflammatory bowel disease (IBD) (9–11) have been brought into renewed focus by the identification of a polymorphism in the activating receptor FcγRIIA that alters susceptibility to ulcerative colitis (UC) (12–14), with subsequent studies demonstrating the pathogenic role of anti-microbial IgG in colitis. In this review, we will address the role that IgG and subsequent Fcγ receptor (FcγR) engagement by local GI-resident immune cells plays in intestinal immunity and inflammation, and the consequence of this interaction for defense against infection, immune maturation, detrimental inflammatory disease, and cancer.

Abbreviations: ADCC, Antibody-dependent cell-mediated cytotoxicity; AHR, Aryl hydrocarbon receptor; AID, Activation-induced cytidine deaminase; APRIL, A proliferation-inducing ligand; BAFF, B cell activating factor; BCR, B cell receptor; CD, Crohn's disease; cDC, Classical/conventional DC; CRP, C reactive protein; DC, Dendritic cell; DSS, Dextran sodium sulfate; FcγR, Fcγ receptor; FcRn, Neonatal Fc receptor; GALT, Gut-associated lymphoid tissue; GI, Gastrointestinal tract; GlcNAc, *N*-acetylglucosamine; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GWAS, Genome wide association study; IBD, Inflammatory bowel disease; IC, Immune complex; IEC, Intestinal epithelial cell; IFN, Interferon; Ig, Immunoglobulin; IL, Interleukin; ILC, Innate lymphoid cell; ITAM, Immunoreceptor tyrosine-based activating motif; ITIM, Immunoreceptor tyrosine-based inhibitory motif; IVIg, Intravenous immunoglobulin; MHC, Major histocompatibility complex; MLN, Mesenteric lymph node; MNP, Mononuclear phagocyte; NOD, Nucleotide-binding oligomerization domain; NET, Neutrophil extracellular trap; NK, Natural killer; pANCA, Perinuclear anti-neutrophil cytoplasmic antibodies; PBMC, Peripheral blood mononuclear cell; PGE2, Prostaglandin E2; PI3K, Phosphatidylinositol3-kinase; pIgR, Polymeric immunoglobulin receptor; PRR, Pattern recognition receptor; RA, Rheumatoid arthritis; REGIIIγ, Regenerating islet-derived protein III-γ; SAP, Serum; SCFA, Short chain fatty acid; SLE, Systemic lupus erythematosus; SNP, Single nucleotide polymorphism; SYK, Spleen tyrosine kinase; TLR, Toll-like receptor; Th, T helper cell; TNF, Tumor necrosis factor; Treg, Regulatory T cell; TRIM21, Tripartite motif-containing protein 21; UC, Ulcerative colitis.

IgG SUBCLASSES AND EFFECTOR FUNCTION

IgG antibodies are the most abundant immunoglobulin isotype in human serum and extracellular tissue fluid, accounting for 10–20% of all plasma protein and 70–75% of total Ig (15). IgG subclasses exhibit diverse effector functions, including the ability to activate complement via the binding and activation of C1q, the engagement of FcγRs on immune cells, and the direct neutralization of toxins and microbes (16). With pleiotropic roles in immunity, detrimental IgG-driven immune responses are associated with several inflammatory and autoimmune disorders, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (17, 18), but IgG antibodies are also key effector molecules that contribute to anti-microbial immunity. Generally, IgG antibodies are known for their high antigen affinity, driven by somatic hypermutation, and are key molecules involved in immunological memory, although these functions vary depending on IgG subclass.

FcγRs are cell surface glycoproteins that bind to the Fc portion of IgG antibodies (19). They are widely expressed across cells of the immune system and are responsible for mediating the cellular effector functions of IgG, including immune cell migration and maturation, the production of inflammatory mediators, and the elimination of opsonized microbes (20). There are several activating FcγRs (FcγRI, FcγRIIA, FcγRIIIA, and FcγRIIIB in humans; FcγRI, FcγRIII, and FcγRIV in mice) and a single inhibitory receptor, FcγRIIB, in both humans and mice, with most exhibiting low-to-medium affinity for IgG (**Figure 1**). The neonatal Fc receptor (FcRn) and the intracellular tripartite motif-containing protein 21 (TRIM21) also bind to immunoglobulins following their internalization (15, 21). FcRn is a major histocompatibility complex (MHC) class I-like molecule that binds to the Fc domain of IgG in a 2:1 stoichiometry with micro- to nanomolar affinity at pH 6.5 within acidic endosomes (22). As well as protecting IgG from degradation with endothelial and myeloid cells, FcRn plays a key role in the active bidirectional transport of IgG across barrier surfaces. It is expressed by murine IECs until weaning and throughout life in the human GI tract. This allows the retrieval of IgG and IgG-antigen immune complexes from the gastrointestinal lumen for phagocytes within the lamina propria, as well as mediating the secretion of IgG (23–26).

For classical FcγRs on the cell surface, productive signaling is initiated by the cross-linking of several receptors into signaling synapses on the cell surface through high-avidity antigen:antibody immune complexes (IC), aggregated IgG, or IgG-opsonized cells and surfaces (**Figure 2**). Upon cross-linking, phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) located on the intracellular domain of activating FcγRs or on the associated common γ-chain (also known as FcεRIγ/FcRγ) occurs. ITAM phosphorylation activates signaling cascades via SRC family kinases and spleen tyrosine kinase (SYK), resulting in downstream activation of phosphatidylinositol3-kinase (PI3K) and phospholipase-Cγ. FcγRIIB contains an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM), which becomes phosphorylated

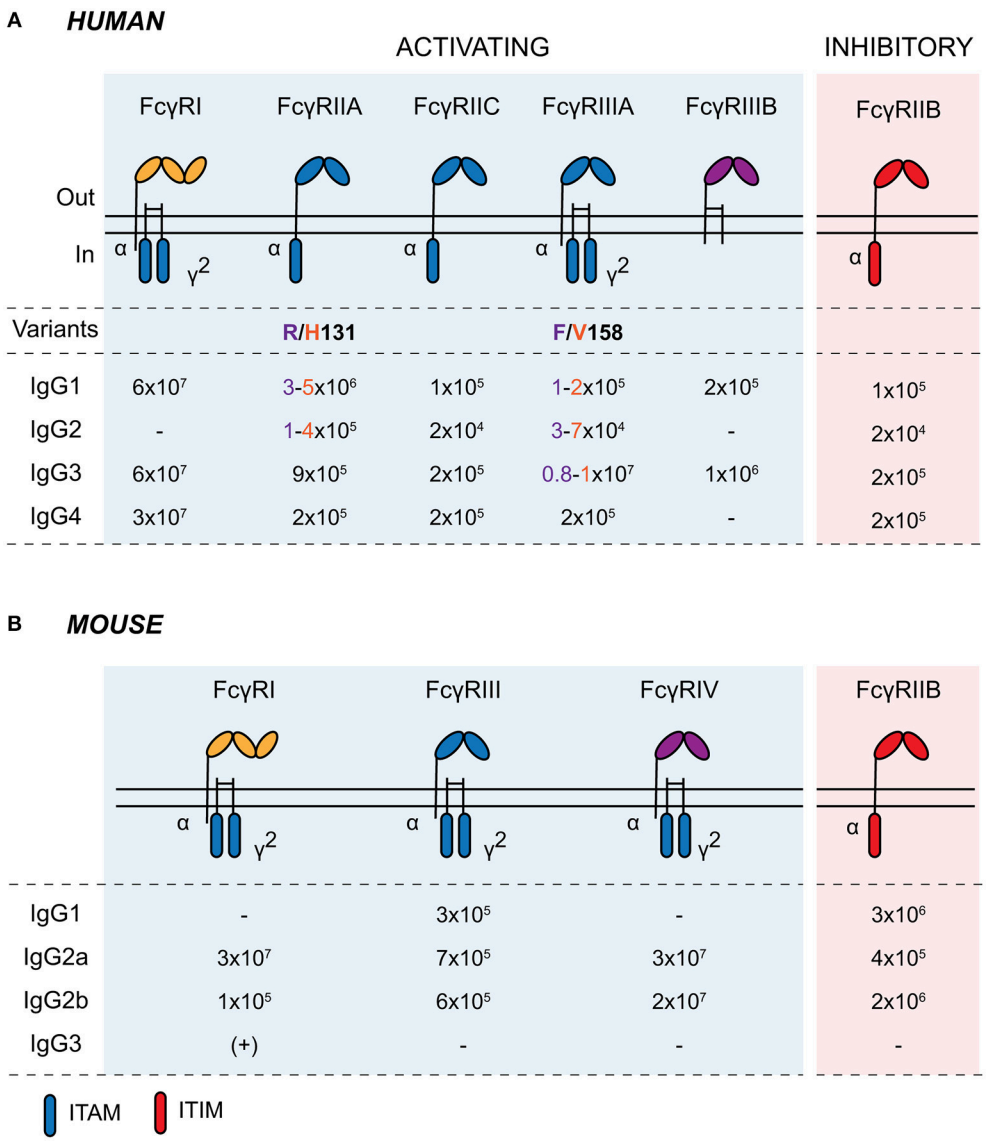

FIGURE 1 | Human and murine Fcγ receptors. Schematic of human **(A)** and murine **(B)** classical Fcγ receptors embedded in the plasma membrane. Activating receptors contain intracellular ITAMs on the intracellular domain of the α chain or in the associated common γ-chain (γ^2; encoded by *FCER1G*). Activating FcγRs are shaded in blue and inhibitory FcγRIIB is shaded in red. IgG affinity-altering variants are highlighted beneath the respective human FcγR, with the low- and high-binding variants and associated IgG affinities colored in purple and orange, respectively, in the table. Binding affinities are indicated as K_A (M^{-1}). ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif.

upon cross-linking with activating FcγRs or the B cell receptor, initiating the recruitment of inositol phosphatases, most notably SHIP1, to the signaling synapse to dampen IgG-mediated responses (18). Activating and inhibitory FcγRs are co-expressed on many immune cells, and their relative expression and activity determines the activation threshold of cells upon encounter of ICs or opsonized targets.

While IgG is the dominant ligand for FcγRs, they can also signal in response to binding to other soluble effectors, such as pentraxins, including C reactive protein (CRP) and serum amyloid P (**Figure 2**) (27, 28). In particular, CRP mediates FcγRIIA-dependent internalization of damaged cells, self-antigens and microbes by human monocytes and neutrophils, via binding to phosphorylcholine moieties, chromatin, and histones (29, 30).

There are four IgG subclasses in humans (IgG1-4) and mice (IgG1, IgG2a/c, IgG2b, and IgG3). Human IgG1 is the most abundant and predominantly targets soluble protein antigens and membrane proteins (15). The generation of IgG1 is largely T cell dependent and it exhibits potent effector function (31). As such, IgG1 is frequently exploited in pharmaceutical settings for the production of therapeutic monoclonal antibodies (32). In mice,

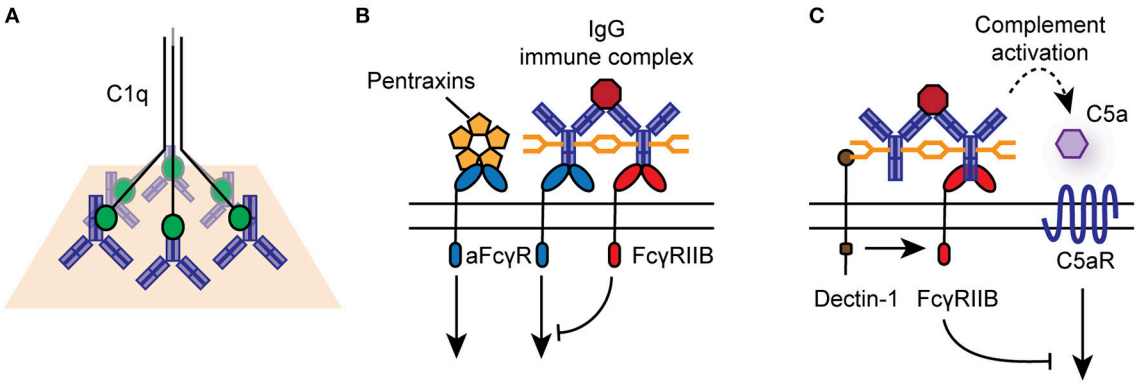

FIGURE 2 | Effector functions of IgG antibodies and FcγRs. **(A)** IgG-opsonized antigens and cells can engage the classical complement pathway via binding to C1q. **(B)** IgG immune complexes, aggregated, or deposited IgG can bind to activating or inhibitory FcγRs on the surface of immune cells, particularly those of the innate immune system. Activating FcγRs (aFcγR) can also bind to pentraxins, such as CRP, linking to cellular responses to innate humoral immunity. **(C)** Specific glycosyl variants of IgG can bind to C-type lectin receptors, such as Dectin-1 and SIGN-R1, to mediated activating FcγR-independent immune function.

the effector profile of IgG2a and IgG2b is most similar to human IgG1 and show the greatest effector function *in vivo* in several settings (17). Human IgG2 responses (IgG3 in mice) are almost completely restricted to T cell-independent bacterial capsular carbohydrates, although anti-carbohydrate IgG antibodies of other subclasses do exist (33). IgG2 and IgG4 antibodies have a short, rigid hinge region compared to IgG1 and 3, resulting in impaired antibody flexibility, and this dictates to a certain extent the affinity of these molecules for FcγRs and C1q. Human IgG3 antibodies are the most effective subclass in terms of their activating effector functions, with enhanced binding to C1q and increased affinity for FcγRs, but they exhibit a blunted half-life due to impaired recycling via the FcRn (34). Finally, IgG4 is associated with induction by allergens following repeated or long-term exposure to antigen in a non-infectious setting, as well as in immune responses to parasitic infections (15). Given its relatively high affinity for the inhibitory receptor FcγRIIB, an ability to spontaneously dissociate and form bispecific antibodies (35), and the capacity to compete with IgE for allergens, IgG4 is often seen as an inhibitor of effector responses (36).

Beyond subclass, IgG effector functions can be fine-tuned through post-translational modification, most notably via *N*-linked glycosylation, which alters antibody stability, FcγR affinity and complement activity (37–40). Each IgG heavy chain carries a single covalently attached biantennary *N*-glycan at the highly conserved asparagine 297 residue in each of the Fc fragment Cγ2 domains, with over 900 IgG glycoforms possible (41). Biantennary complexes can contain additional bisecting *N*-acetylglucosamine (GlcNAc), core fucose, galactose and sialic acid residues (42).

Defucosylated IgG exhibits enhanced FcγRIIIA affinity (43), while sialylation promotes anti-inflammatory functions of IgG by reducing FcγR affinity and promoting binding to the C-type lectin receptor, SIGN-R1 (DC-SIGN in humans) (37, 44, 45). Indeed, sialylation is required for some of the protective functions of intravenous immunoglobulin (IVIg). Agalactosylated IgG, with two oligosaccharide chains ending in GlcNAc rather than galactose/sialic acid, is termed G0 (no galactose) (19). G0 IgG can activate complement via binding to mannose binding lectin and can bind the mannose receptor on phagocytes (46, 47).

In summary IgG antibodies are powerful effector molecules that can mediate tissue inflammation by complement activation, engagement of classical FcγRs and C-type lectin receptors (**Figure 2**). They are the dominant circulating antibody in humans and mice, with documented roles in defense and autoimmunity but their contribution to immunity in the gastrointestinal tract is much less well-defined.

IMMUNITY IN THE GASTROINTESTINAL TRACT

The frontline barrier between the external environment in the lumen of the GI tract and host tissues is the intestinal epithelium. This physical and biochemical barrier consists of an outer-most layer of thick secreted mucus, a single-cell layer of IECs, and the underlying non-epithelial mucosal cells, including a network of leukocytes found within the lamina propria, muscularis layers, and organized lymphoid follicles (48).

The largest mucosal barrier in the human body, the intestinal epithelium is continuously renewed by pluripotent intestinal epithelial stem cells located in the base of the crypts in a manner regulated by the local stem cell niche (49). Although absorptive enterocytes are the most abundant cell subset, IECs form a heterogeneous network of cells with distinct functions. For example, secretory Paneth cells and goblet cells produce anti-microbial peptides and mucins, respectively, while M cells transcytose antigens across the intestinal epithelium to the Peyer's patches beneath. Enterocytes also produce a restricted repertoire of anti-microbial peptides throughout the GI tract, such as the C-type lectin regenerating islet-derived protein III-γ (REGIIIγ). The ability of IECs to form an effective barrier depends on their ability to act as frontline sensors of microbial stimuli through their expression of pattern recognition receptors

(PRRs), including Toll-like receptor (TLR)5 and nucleotide-binding oligomerization domain (NOD)-like receptors (50–52). In turn, IEC-derived cytokines play essential roles in shaping the microbial structure of the GI tract, as well as the activation state of local immune cells during both homeostasis and inflammation (50, 53, 54). For example, interleukin (IL)-18 (50), IL-25 (55), thymic stromal lymphopoietin (56), and B cell-stimulating factors, including a proliferation-inducing ligand (APRIL) (57), are induced by PRR signaling in IECs and act to regulate local immunity. The importance of the epithelium in intestinal health is illustrated by genetic polymorphisms linking epithelial health and effector function with susceptibility to IBD and systemic inflammatory diseases, as shall be discussed later (58, 59).

Beneath the epithelium, the intestinal mucosa and submucosa houses a range of innate and adaptive immune cells (**Figure 3**). Tissue-resident macrophages and dendritic cells (DCs) promote tolerance to intestinal commensal via anti-inflammatory cytokine production (including IL-10, and TGFβ, see section below). In addition, there are substantial populations of intestinal T cells, and the balance between regulatory T cells (Tregs) and T helper (Th)17 cells plays a fundamental role in determining whether there is mutualism with commensals or if their presence provokes inflammation. This is of particular interest, given the association of dysregulated IL-23/type 17 responses in the pathology of IBD and their role in defense against enteropathogens, such as *Citrobacter rodentium* (60–65).

IL-17A production by Th17 cells, γδ T cells and group 3 innate lymphoid cells (ILC3s) at epithelial barriers sites can play a central role in intestinal inflammation (66–69). IL-17 promotes neutrophil recruitment to tissues via the induction of CXCL1, CXCL2, and CXCL8, while IL-17A-induced granulocyte-macrophage colony-stimulating factor (GM-CSF) and matrix metalloprotease can sustain neutrophil activation and survival (70–72). IL-17 can also induce expression of anti-microbial peptides (73) and promotes the maintenance of the intestinal epithelial barrier (74–76), therefore IL-17 has both pro-inflammatory and homeostatic roles. The IL-17-inducing cytokine IL-23 can also stimulate interferon (IFN)γ secretion by inflamed colonic lamina propria cells (77), which in turn contributes to inflammation through the activation of tissue-resident macrophages, apoptosis of IECs, and augmentation of antigen processing (63). Studies utilizing IFNγ-deficient mice or IFNγ-depletion confirm its contribution to intestinal inflammation in murine models (78–80).

IL-10 is the prototypical anti-inflammatory cytokine. Most classically associated with Foxp3$^+$ Tregs, IL-10 functions to suppress lymphocyte and myeloid immunity through a variety of mechanisms. Within the GI tract, the critical role of this cytokine is acutely illustrated by associations between *IL10R* single nucleotide polymorphisms (SNPs) and UC, as well as the widespread use of the *Helicobacter hepaticus* infection model: the presence or absence of IL-10R signaling dictates whether an otherwise tolerated bacterium induces colitis (81–83). Beyond Tregs and macrophages, regulatory B cells and epithelial cells also contribute to the intestinal IL-10 pool (84–86). Indeed, Rosser et al. demonstrated that microbiota-dependent IL-6 and IL-1β production was sufficient to induce IL-10-producing B cells within mesenteric lymph nodes (MLNs) that contain systemic inflammation (85), while adoptive transfer of these cells is sufficient to suppress intestinal inflammation (86).

ANTIBODIES IN THE GASTROINTESTINAL TRACT

IgA in the GI Tract

While constituting only 10–15% of total serum Ig, IgA is the major antibody isotype at mucosal surfaces (87). Of the two major isoforms in humans (IgA1 and 2), IgA2 is associated with mucosal sites and has enhanced resistance to proteolysis. The intestinal epithelium has the capability of transporting of Ig into the lumen via expression of the polymeric immunoglobulin receptor (pIgR). Following pIgR binding and internalization at the basolateral IEC surface, dimeric IgA is released at the apical membrane by proteolytic cleavage of the secretory component of pIgR, forming secretory IgA. Once within the lumen, secretory IgA participates in the maintenance of mutualism through mechanisms collectively known as *immune exclusion*. This includes the ability to neutralize toxins and pathogens in the absence of complement fixation (88), the anchoring and agglutination of microbes within the mucus layer (89), as well as the recently described ability to "enchain" dividing bacterial daughter cells to limit microbial colonization and evolution, and promote elimination from the body (90). Natural or low-affinity IgA can also deliver antigens to M cells for transport into Peyer's patches for the induction of adaptive immune responses (91).

Gut-draining MLNs, gut-associated lymphoid tissues (GALT), including Peyer's patches and isolated lymphoid follicles, and the intestinal lamina propria itself have been proposed to be sites for IgA generation, with the mode of IgA generation differing across tissues (87, 92). Local IgA production within the lamina propria and isolated lymphoid follicles is thought to be largely T cell-independent, given the absence of abundant T cell zones, instead relying on B cell-intrinsic TLR signaling and secretory factors released by DCs and non-haematopoietic cells (57, 93). The MLNs, caecal patch, and Peyer's patches can support both T cell-dependent and -independent IgA induction, with the former linked to invasive species of commensals and pathobionts (94–97).

IgG in the GI Tract

IgG1$^+$ and IgG2$^+$ plasma cells are present in the human gut. Both polyreactive and antigen-specific responses have been identified that target both foreign (commensals and enteropathogens) and self-antigens (10, 98). All antibodies show evidence of antigen-mediated selection and somatic hypermutation, with little difference in antigen reactivity between IgG isotypes, and no evidence of clonal relation between IgG and IgA responses within donors.

IgG2b and IgG3-producing B cell populations were also identified in the Peyer's patches and MLN of healthy adult mice, consistent with human observations. Murine IgG was elicited primarily by commensal-driven TLR signaling on B cells in a T cell-independent manner. Despite the relatively lower concentration of mucosal IgG compared with IgA, intestinal

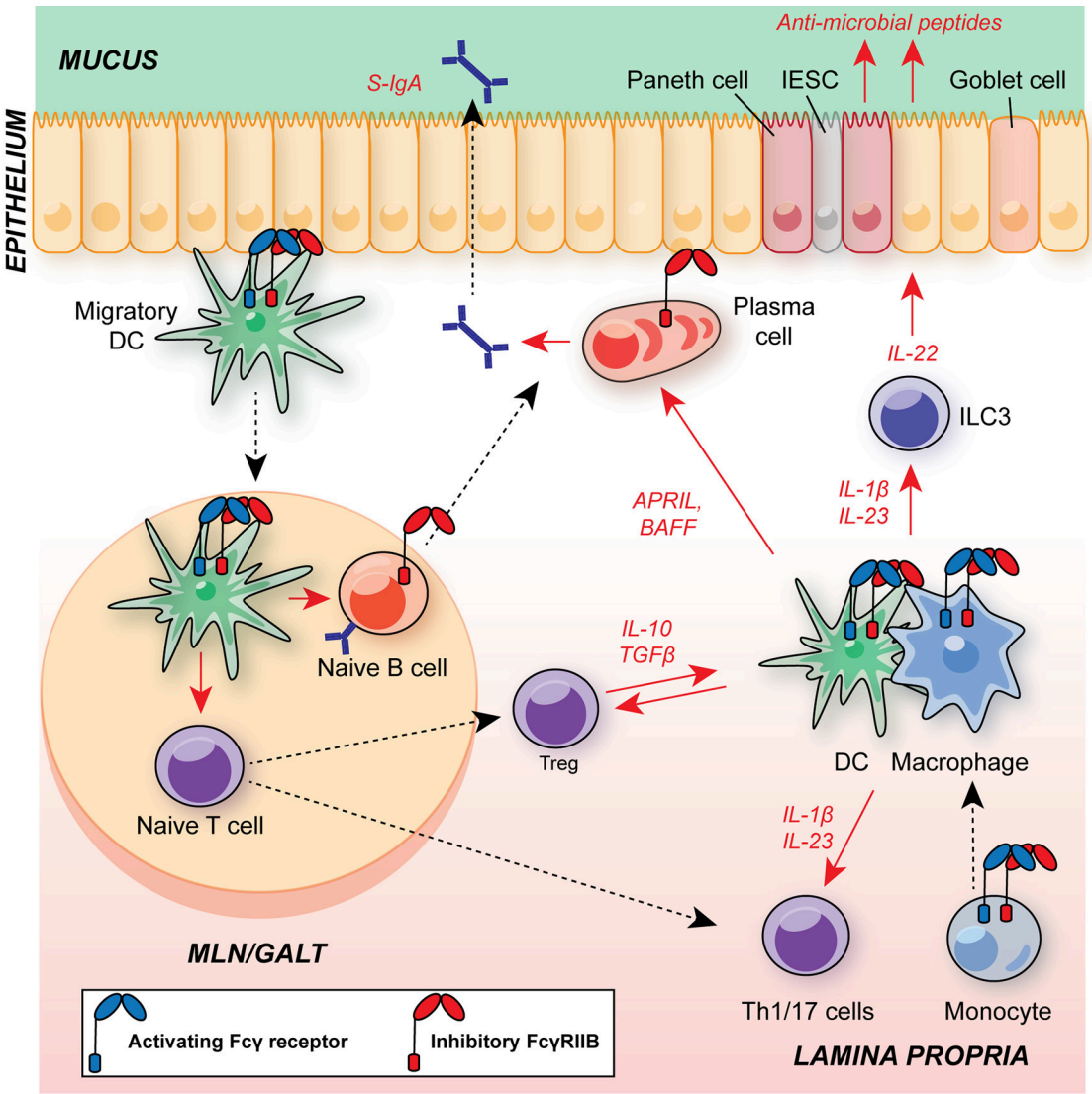

FIGURE 3 | Schematic of major leukocyte populations in the intestine during homeostasis and their expression of FcγRs. Migratory DCs promote B cell IgA class-switching and T cell polarization in GALT and MLNs, including regulatory T cells (Treg) and Th17 cells. The balance of Tregs and Th17 cells is critical for maintaining intestinal homeostasis and suppressing excessive responses to the microbiota. CD11b^{+} cDC2s have an A:I ratio skewed toward FcγRIIB expression, as observed in DCs in other organs. FcγR-expressing DCs and monocyte-derived macrophages within the lamina propria support T cell and ILC3 activation via commensal-dependent production of cytokines, including IL-1β and IL-23. Tissue-resident macrophages are dependent on IL-10 for their suppressive function. APRIL and BAFF production by DCs and macrophages supports local plasma cell survival. Macrophage A:I ratio is skewed toward activating FcγRs and tuneable to the local milieu. Plasma cells and B cells express FcγRIIB, regulating survival and BCR activation threshold in these cells, respectively. Blue receptor, activating FcγR; red receptor, inhibitory FcγRIIB; S-IgA, secretory IgA; MLN, mesenteric lymph node; GALT, gut-associated lymphoid tissue; DC, dendritic cell; APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor; IESC, intestinal epithelial stem cell; ILC3, group 3 innate lymphoid cell.

IgG responses exhibit broad microbial reactivity. Furthermore, most commensals bound by IgA antibodies in the murine gut also engage serum IgG2b and IgG3, while certain species of commensal microbes are uniquely opsonized by IgG3 across bacterial phyla (7).

The signals that lead to the local generation of IgG within the gastrointestinal tract have not been completely defined. However, *Escherichia coli* LPS has been shown to induce the B cell enzyme activation-induced cytidine deaminase (AID) and T cell-independent class-switch recombination to IgG2b and IgG3 in mice through dual B cell receptor (BCR)/TLR4 signaling (99, 100), and this may contribute to IgG generation in GALT. Indeed, a subset of IgG3- and IgG2b-expressing B cells within the Peyer's patches and MLN express the B1 cell marker CD43, a B cell subclass specializing in T cell-independent IgG production (7). T cell-dependent IgG responses have also been reported from B2 and follicular B cells in the GI tract, although the nature of the signals dictating T cell dependency remains to be determined.

Higher affinity T cell-dependent responses may be reserved for invasive or epithelium-adherent species, in a manner analogous to IgA (90, 101) and consistent with the role of immune IgG in protection from enteropathogens (6, 102). Natural antibodies of the IgG3 subclass have also been described in mice and shown to interact with ficolins and mannose binding lectin that bind to bacteria, leading to FcγR-mediated phagocytosis by monocytes and inflammatory cytokine production (103).

Fcγ Receptors and Their Expression in Immune Cells in the GI Tract

FcγRs are expressed by a number of immune cells within the GI tract, conferring the ability to respond to local IgG IC, and we will consider each cell type in turn:

Intestinal Macrophages

Macrophages play a central role in tissue homeostasis and inflammation, with increasingly appreciated non-immune roles in tissue function. Within the GI tract, macrophages are found throughout the lamina propria, the connective tissue that underlies the intestinal epithelium, as well as between the circular and longitudinal muscle layers (104–106). Here, they are continuously exposed to microbial stimuli and play a central role as key guardians of the homeostatic milieu through their potent phagocytic capacity and ability to produce an array of cytokines and chemokines.

Elegant studies by Bain et al. demonstrated that adult intestinal macrophages are largely derived from CCR2-dependent Ly6C^{hi} monocyte recruitment, a process dependent on the microbiota (107). Although intestinal macrophage numbers are unaltered in germ-free mice (108), commensal microbes influence macrophage activation state (109), for example via the production of short chain fatty acids (SCFAs) (110).

In homeostasis, intestinal macrophages are skewed toward anti-inflammatory functions, including the basal production of IL-10 (104, 111, 112). Indeed, macrophage-derived IL-10 can dampen IL-23-mediated colitis and promote regulatory T differentiation *in vitro* (111, 113).

During colitis, infiltrating monocytes are redirected toward a more pro-inflammatory macrophage phenotype (112, 114), with elevated production of IL-1β, IL-23, and tumor necrosis factor (TNF). These, in turn, promote Th1/Th17 and innate lymphoid cell (ILC) activation. Thus, inflammatory macrophages are the principle mediators of tissue inflammation in several murine models of intestinal pathology (60, 115–119).

Intestinal macrophages express a number of cell surface receptors, including TLRs, C-type lectin receptors, NOD-like receptors, and FcγRs (120). Indeed, FcγRI (CD64) is often used to discriminate between macrophages (FcγRI$^+$) and dendritic cells (FcγRI$^-$) in the GI tract (121). Studies in human monocyte-derived macrophage and murine bone marrow derived macrophages demonstrate expression of all canonical FcγRs, and expression is tuneable to the local environment: Th2 cytokines, such as IL-4, upregulate the expression of FcγRIIB and decrease the so-called activating/inhibitory ratio on macrophages (122–124). Generally, however, FcγR expression is skewed in favor of activating signaling (125, 126). A comprehensive analysis of FcγR expression in human gastrointestinal macrophages, across different anatomical regions of the gut, and in health and disease has yet to be performed.

FcγR cross-linking *in vitro* induces a potent macrophage inflammatory response, characterized by the production of IL-1β, IL-6, IL-10, IL-12, and TNF-α, as well as chemokines including CXCL8 (127). Interestingly, several studies have highlighted a link between TLR and FcγR co-stimulation and the induction of a Th17 polarizing macrophage phenotype. Stimulation of human M2 macrophages and DCs with IgG-opsonized bacteria induces IL-1β and IL-23 expression (128–130). Given the role of these same macrophage-derived cytokines in intestinal pathology, FcγR cross-linking by anti-commensal IgG signaling could contribute to Th17 cell activation and inflammation in the human gut. However, some murine studies suggest that IgG immune complexes can inhibit LPS-induced IL-1β and TNF production in BMDMs, a phenomenon at least partly dependent on prostaglandin E2 (PGE2) production (131, 132). Therefore, further work is needed to understand whether *in vitro*-derived macrophages accurately reflect tissue macrophage activation by IgG immune complexes *in vivo*, particularly in the complex environment of the gastrointestinal tract.

Intestinal Dendritic Cells

DCs sit at the interface of innate and adaptive immunity, their primary function being the transport of antigen from peripheral sites to draining lymph nodes in a CCR7-dependent manner for MHC-dependent presentation to T cells. Additionally, DC-derived cytokines play key roles in shaping innate and adaptive immune responses, including T cell polarization, B cell class-switch recombination, and innate lymphoid cell activation (**Figure 3**) (105, 133).

There are two main subsets of classical or conventional DCs (cDCs) derived from DC-specific precursors: BATF3-dependent cDC1s and IRF4-dependent cDC2s (134). In the murine intestine, several DC subsets have been characterized that variably express CD11b and CD103, with unique functions in health and disease (105). CD103$^+$ CD11b$^-$ cDC1s predominantly reside within GALT and are specialized in cross-presentation, while CD103$^+$ CD11b$^+$ cDC2s are found within the lamina propria and migrate to the MLN for presentation of exogenous antigens to CD4$^+$ T cells and promote humoral immunity. CD103$^+$ CD11b$^+$ cDCs are essential for IL-6-dependent Th17 generation in the small intestine and colon (135) and necessary for the generation of IgG- and IgA-class-switched B cell responses to flagellin in MLNs (136). CD11b$^+$ CD103$^-$ DC subsets have also been described with the capacity to induce IL-17A and IFNγ production by T cells (137, 138). CD103$^+$ DCs have also been identified as major sources of IL-23 in the context of *Citrobacter rodentium* infection, a widely used model of human attaching-effacing *Escherichia coli* infection (139–141). CD103$^+$ DCs are also essential for the maintenance of tolerance via the induction of gut-homing CCR9$^+$ FoxP3$^+$ Tregs and IgA$^+$ plasma cells, both within MLNs and GALT (88, 97, 133, 142).

DCs express FcγRI and FcγRIIA in humans, and FcγRIII in mice, although FcγR expression is skewed toward inhibitory FcγRIIB in immature DCs. Indeed, FcγRIIB blockade leads to spontaneous DC maturation and the induction of a cytokine programme characterized by TNF, IL-6, CXCL8, and IL-12p70 production (127, 143, 144), although as with macrophage studies, these data are generated on *in vitro* differentiated DCs. Analysis of Immgen consortium datasets on *in vivo* differentiated DCs identifies FcγR expression with cDC2s (126). This expression is tightly regulated, with maturation signals, such as LPS or IFNγ, down-regulating FcγRIIB to allow for IgG-induced cell activation via activating FcγR ligation. DCs efficiently process FcγR-internalized antigen and upregulate MHC and co-stimulatory molecules for robust antigen presentation to T cells (145–147). Furthermore, FcγR cross-linking on DCs induces CCR7 and matrix metalloprotease expression that facilitates their migration from inflamed peripheral sites to local draining lymph nodes (148). As well as its role in IgG recycling, FcRn has also been demonstrated to mediate the presentation of immune complexed antigens by DCs; FcRn engagement by immune complexes protects antigens from degradation following FcγR-mediated internalization, preserving them for presentation and cross-presentation to CD4 and CD8 T cells, respectively (149). Therefore, classical FcγRs and FcRn play a key role in DC antigen presentation to T cells through a variety of mechanisms.

As with macrophages, comprehensive information on FcγR expression in human gastrointestinal DCs across different anatomical regions of the gut is currently lacking.

Intestinal Neutrophils

The most abundant circulating leukocyte, neutrophils are first line inflammatory responders specializing in anti-microbial defense. This includes microbial internalization and killing, the release of proteases and reactive oxygen species, cytokine production, and the formation of neutrophil extracellular traps (NETs) (150). These functions are critical for defense following barrier breach, but their potent pro-inflammatory capability inevitably means that neutrophils may also contribute significantly to collateral tissue damage during inflammatory responses. Beyond microbicidal activity, neutrophils can shape adaptive immunity, for example via the production of APRIL and B cell activating factor (BAFF) in the marginal zone of the spleen (151), and may even be able to present antigen to T cells (152–154) identifying neutrophils as a more versatile immune subset than previously appreciated.

Neutrophils have long been associated with inflammation in patients with IBD and in defense against enteropathogens, rapidly recruited to the mucosa by resident and inflammatory mononuclear phagocytes (MNPs) (155, 156). However, their role in intestinal inflammation remains enigmatic, exhibiting both detrimental and protective functions in a context-dependent manner (157–159), including the production of barrier-protective IL-22 (160). The extravasation of neutrophils into the intestine is also observed during homeostasis, where their engulfment by resident MNPs suppresses *Il23a* induction (161).

Neutrophils constitutively express FcγRIIA and FcγRIIIB in humans, and FcγRIII and FcγRIV in mice, with low levels of FcγRIIB in both species (162–164). FcγR cross-linking has profound effects on neutrophil function, as expected of cells acutely poised to respond to stress. This includes NET formation (165), cytokine production, such as IBD-associated TNF and oncostatin M (166, 167), endothelial cell adhesion (168), phagocytosis (169), and reactive oxygen species production (170). Unsurprisingly, therefore, polymorphisms affecting FcγR function have consequences for the IgG-mediated activation of neutrophils. Most notably, three variants of FcγRIIIB exist (NA1, NA2, SH) that exhibit differential activity. NA1 is associated with enhanced uptake of IgG-opsonized erythrocytes (171), while SH enhances surface FcγRIIIB expression (172). Furthermore, copy number variation in *FCGR3B* correlates with FcγRIIIB surface levels, IC uptake, and soluble serum FcγRIIIB (164).

B Cell Response

As well as being the source of antibodies, B cells are also themselves regulated by IgG via expression of FcγRIIB, where it cross-links to the BCR to increase the cellular activation threshold and suppress antibody production (18). Furthermore, direct cross-linking of FcγRIIB on the surface of mature B cells and bone marrow-resident plasma cells can directly mediate apoptosis, thereby limiting the peripheral pool of antibody-producing cells (173, 174). As such, FcγRIIB has a critical role in maintaining humoral tolerance. Whether local IgG-IC engagement of FcγRIIB in GALT similarly regulates B cells in the gastrointestinal tract is currently undetermined.

Innate Lymphocytes

ILCs are a recently identified class of immune cells that are enriched at mucosal sites, such as the GI and respiratory tracts (175). ILCs subsets mirror those seen for T cells. Natural killer (NK) cells represent the innate cytotoxic equivalent of $CD8^+$ T cells, whereas non-cytotoxic *helper* ILCs resemble $CD4^+$ T helper cells. All helper ILCs, hereafter referred to simply as ILCs, express IL-2Rα and IL-7Rα, but unlike T cells and B cells, they do not express somatically-rearranged antigen-specific receptors (176). Helper ILC subsets are divided into ILC1s, ILC2s, and ILC3s that largely mirror the transcription factor-dependence and cytokine effector profile of Th1, Th2, and Th17 cells, respectively.

Given the importance of Th17 biology in the gut, it is unsurprising that the GI tract represents one of the major sites of ILC colonization. Mature $NKp46^+$ ILC3s reside primarily in the intestinal mucosa and require RORγt for their development (177–179). Lymphoid tissue inducer (LTi) cells primarily reside within SLOs and represent a distinct ILC3 subset that develops partly independently of other helper ILCs (180). They contribute to lymphoid organogenesis and regulate local immunity through MHC-II expression and cytokine production (181–184). Through their production of IL-22, ILC3s play a critical role in the reinforcement of the intestinal epithelial barrier. Early studies demonstrated that *C. rodentium* infection induced innate intestinal IL-22 production that was dependent on IL-23 and the microbiota (117, 185). Subsequently, several studies have shown that IL-22R signaling mediates REG family AMP production, IEC fucosylation, and epithelial stem cell proliferation (64, 186–191). ILC3s are also an important source of GM-CSF, a key regulator

of tissue immunity. In complementary studies, ILC3-derived GM-CSF was shown to orchestrate monocyte and granulocyte recruitment to the inflamed large intestinal lamina propria in anti-CD40 and *H. hepaticus* models of colitis (192–194).

FcγR expression has not been extensively investigated in ILCs. Their cytotoxic counterparts, NK cells, express activating FcγRIIC and FcγRIIIA in humans, and FcγRIII in mice, but not FcγRIIB (195, 196). FcγR signaling on NK cells stimulates the targeted release of cytotoxic molecules to kill opsonized cells, a process known as antibody-dependent cell-mediated cytotoxicity (ADCC), as well as IFNγ and TNF-α release (197, 198). Several lines of evidence suggest that FcγR signaling may impact ILC function, either directly or indirectly. Firstly, FcγR signaling on MNPs may drive ILC3 activation through the production of type 17-inducing cytokines, such as IL-1β and TL1A (129, 199, 200). Secondly, FcγRIII expression has been identified in murine ILC3s in global transcriptomic studies (201, 202). Therefore, FcγR expression may be a common feature of cytotoxic and helper ILC subsets.

INTESTINAL IgG IN HEALTH AND PATHOGEN DEFENSE

Neonatal Immunity

The acquisition of maternally-derived secretory antibodies *in utero* and through breast milk provides neonates with an important source of immunity prior to the development of the host immune system (203). Mucosal pathogens are a major cause of death in children below the age of 5, with epidemiological data indicating that breastfeeding confers a 20-fold reduction in infant mortality from diarrhea. In this setting, maternally-derived IgG plays a key role in the maintenance of mutualism between the neonate and microbiome, tolerance toward innocuous dietary and environmental antigens, and systemic protection from pathogenic challenge (5–8, 204, 205).

Koch et al. demonstrated that in the absence of intestinal FcRn-mediated IgG uptake, neonates exhibited enhanced bacterial translocation to the MLN and compensatory mucosal inflammatory responses, most prominently exacerbated T follicular helper responses and germinal center B cell activation (**Figure 4**) (7). This response was subsequently superseded by *de novo* anti-microbial IgG generation after weaning. Similarly, maternally-derived intestinal IgG was demonstrated to mediate protection against *Heligmosomoides polygyrus*, both through luminal delivery via milk and FcRn-mediated epithelial transport from the circulation (206). In a model of commensal colonization, Macpherson and colleagues further demonstrated the requirement for maternal IgG antibodies in the development of tissue-resident immune cells in neonates. Neonatal expansion of small intestinal NKp46^{+} ILC3s and IL-22-dependent genes, such as REGIIIγ, was IgG-dependent, driven by the retention and subsequent transfer of microbe-derived metabolites to offspring as immune complexes (5) (**Figure 4**). Strikingly, this included ligands for AHR, a key transcription factor mediating ILC3 development (178, 207). Consequently, offspring to colonized dams were better able to control intestinal microbial challenge and suppress systemic dissemination of bacteria.

Beyond microbial recognition, intestinal IgG plays a key role in tolerance to innocuous antigens. In a recent elegant study, Ohsaki et al. demonstrated that epicutaneous sensitization of pregnant mothers with model allergens resulted in suppressed allergic responses in offspring subsequently challenged with the same antigen (8). Lower allergen-specific IgE titres, impaired mucosal and systemic Th2 activation, and reduced anaphylaxis was attributed to intestinal FcRn-mediated allergen:IgG immune complex uptake and presentation within MLNs by tolerogenic DCs which, in turn, induced protective mucosal Tregs. Indeed, given the roles for FcγR signaling in DC activation, this suggests IgG-mediated trafficking and presentation may be a common mechanism of immune complex-mediated immune responses in DCs across tissues (148). Whether similar mechanisms exist for the induction of microbe-induced Tregs remains to be established. Furthermore, how DC presentation of IgG-opsonized antigens may be altered during mucosal inflammation, and whether this induces pro-inflammatory T cell responses that promote microbial elimination has yet to be explored.

Intestinal and Systemic Infection

In healthy adult mice, microbe-specific IgG is largely excluded from the intestinal lumen, suggesting that its major site of action is in the intestinal wall, where it combats invasive microbial species, and in protection from systemic challenges. For example, IgG responses induced by gram negative bacterial antigens have been demonstrated to confer FcγR-mediated protection against systemic *E. coli* and *Salmonella* challenge (205). Indeed, murine IgG2b is known to effectively engage FcγRs and complement receptors, suggesting a role in the activation of mucosal phagocytes (208). A striking aspect of this response is its inherent flexibility, tuneable to fluctuations in microbial loads and to genetically-determined variation in the strength of the mucosal innate immune system. For example, defective TLR signaling or oxidative burst production augments gut T cell-dependent microbial IgG titres that act to preserve mutualism *in vivo* (204). Therefore, IgG-mediated anti-commensal responses represent an essential and plastic mechanism required to maintain host-microbe mutualism and mediate protection from systemic pathogen spread.

The context of antigen delivery to GALT is a critical determinant of subsequent humoral immune responses. Antigen presentation in inflamed or infected mucosae, or from high antigenic loads, induces potent antibody responses due to alterations in adjuvant-derived signals, leading to high-avidity T cell-dependent antibodies that contribute to specific elimination of the inductive bacterial species (90, 209). As well as being induced continuously in GALT largely independently of T cells, *de novo* T cell-dependent IgG has been identified in models of intestinal infection, where it has been shown to make essential contributions to sterilizing immunity.

In murine models, B cells are required for clearance of *C. rodentium*, although this was independent of secretory IgA and IgM (102). Although initial disease activity and bacterial containment was equivalent between B cell-sufficient

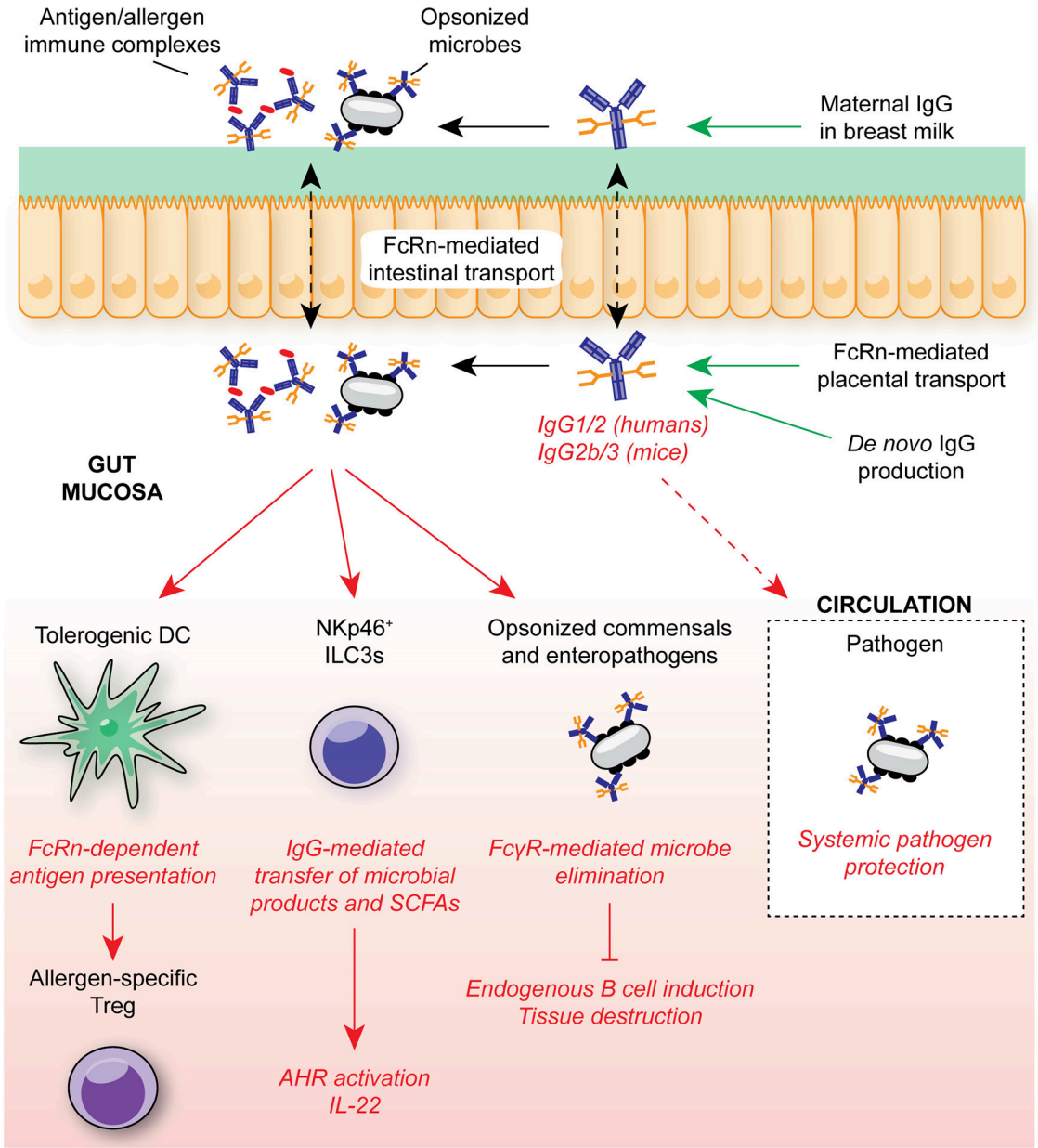

FIGURE 4 | IgG in intestinal homeostasis. FcRn-mediated placental and epithelial transport contributes to the neonatal anti-microbial IgG repertoire in early life, mediating protection against opportunistic mucosal invasion. Maternally-derived IgG contributes to protection from allergic responses through FcRn-mediated antigen presentation by DCs to T cells for regulatory T cell induction. IgG-mediated transfer of microbial molecules, such as SCFAs, also supports appropriate immune cell development. In adult humans and mice, anti-microbial IgG is generated throughout life in GALT, contributing to systemic protection from infection through engagement of FcγRs on myeloid cells. In humans, FcRn is continuously expressed within the intestinal epithelium, allowing for bidirectional trafficking of IgG and immune complexes between the intestinal lumen and lamina propria for antigen delivery to local FcγR-expressing myeloid cells. SCFA, short-chain fatty acids; ILC3, group 3 innate lymphoid cell; FcRn, neonatal Fc receptor; AHR, aryl hydrocarbon receptor.

and deficient mice at 2 weeks post-infection, B cell-deficient mice exhibited enhanced mucosal inflammation and severe crypt hyperplasia with ulceration at 6 weeks, consistent with defective infection control. Disease susceptibility was rescued only by passive transfer of IgG-replete immune serum (210). Anti-*C. rodentium* IgG titres are diminished in $CD4^+$ T cell-deplete mice, which are highly susceptible to *C. rodentium*, suggesting a critical role for T cell-dependent antibody responses (211). Adequate IgG responses to *C. rodentium* are also dependent on NOD2-mediated bacterial sensing within the intestinal epithelium. Impaired local adaptive immune activation is observed in *Nod2*-deficient mice, attributed to reduced CCL2-mediated monocyte

recruitment (212). Monocyte-derived IL-12 was required for induction of Th1 cells (213), with IFNγ being an effective driver of IgG class-switching (214, 215), suggesting a possible mechanism for this effect. As well as complement-fixing activity (216), the contribution of FcγRs to IgG-mediated protection in this model was investigated using Fc receptor common gamma chain (FcRγ)-deficient mice, which lack productive signaling from activating FcγRs (217). FcRγ-deficient mice phenocopy B cell-deficient mice, succumbing more rapidly to infection, with increased bacterial burden and mucosal inflammation. In the absence of FcγR signaling, MNP-mediated *C. rodentium* phagocytosis, cellular maturation, and inflammatory cytokine production was impaired, as was antigen presentation to T cells. In an elegant study, Kamada et al. demonstrated the specific targeting of virulence factors on invasive strains of *C. rodentium* by mucosal IgG, consistent with their ability to adhere to the intestinal epithelium and elicit inflammation (6). In contrast, non-virulent strains and commensals residing predominantly within the lumen remained untargeted. Both MNPs and neutrophils are required for FcγR-mediated protection, with neutrophils shown to directly access opsonized bacteria within the intestinal lumen (6). Furthermore, more recent work from Caballero-Flores et al. has demonstrated that this IgG-mediated protection against *C. rodentium* can be vertically transmitted to offspring via breast milk (218).

In humans, FcRn expression is not limited to a brief developmental window, allowing the entry and retrieval of IgG from the intestinal lumen throughout health and disease. The importance of this was demonstrated in transgenic murine experiments with forced expression of human FcRn in murine IECs (26). This bidirectional transport allowed the secretion of IgG into the lumen, the subsequent uptake of opsonized bacteria, and the induction of local antigen-specific $CD4^+$ T cell responses required for clearance. Therefore, cross-talk between $CD4^+$ T cells and IgG-expressing B cells appears to be required for effective intestinal pathogen clearance. FcRn-mediated protection has also been demonstrated in a mouse model of *Helicobacter pylori* infection, with reduced IgG levels in gastric juice of challenged FcRn-deficient animals, with increased bacterial penetrance and activated lymphoid follicles compared to controls (219).

Beyond models of murine bacterial challenge, several studies in non-human primates have also identified roles for IgG in anti-viral mucosal immunity. Passive administration of anti-HIV neutralizing IgG can prevent mucosal viral transmission in rhesus macaques following oral administration (220, 221). The Fc domain of anti-HIV broadly neutralizing antibodies was required for anti-viral activity *in vivo* (222) and could be further enhanced through Fc domain engineering to augment Fc-mediated activating FcγR engagement (222). Fc domain modification to increase FcRn binding also increased the serum half-life of the antibody and enhanced mucosal tissue localization (223). In a recent study, it was demonstrated that the site of immunization influences the dominant protective mechanisms of elicited Igs (224). Intramuscular vaccination induced an IgG-dominated response, with protection correlating most strongly with FcγR-mediated viral phagocytosis by monocytes. In contrast, mucosal vaccination via aerosol elicited an IgA-skewed response that correlated with neutrophil-mediated phagocytosis. Protection via mucosal vaccination also correlated significantly with FcγRIIA binding, suggesting that mucosal vaccine-specific IgA and IgG may cooperate to drive neutrophil-mediated viral clearance *in vivo*. Despite these differences in mechanism, both routes were equally effective in suppressing viral infection. Similarly, passive transfer of serum rotavirus-specific IgG has been shown to suppress oral rotavirus infection in naïve pigtailed macaques (225).

IgG, FcγRs, AND INTESTINAL INFLAMMATION

Inflammatory Bowel Disease

Inflammatory bowel disease is a chronic relapsing inflammatory disorder of the GI tract that causes considerable morbidity and is associated with an increased risk of colonic cancer (156, 226, 227). There are two main subtypes, Crohn's disease (CD), and UC, that differ in their clinical and pathological presentations. CD may affect any part of the GI tract and is associated with transmural inflammation affecting the entire mucosa and the formation of granulomas. In contrast, in UC, lesions are localized to the large bowel, resulting in continuous superficial mucosal inflammation and ulceration of the intestinal wall, with micro-abscess formation and neutrophil infiltration within the lamina propria common.

GWA studies (GWAS) have contributed significantly toward the understanding of the pathogenesis of IBD, with the immune system and its interaction with the microbiome lying at the core of disease susceptibility. Disease is driven by a genetic predisposition to aberrant mucosal immune responses toward the microbiota in the context of poorly-defined environmental factors, with unique and shared genetic traits between CD and UC (156). Furthermore, many disease-associated mechanistic pathways in IBD are shared with other extra-intestinal diseases, including ankylosing spondylitis and psoriasis (14, 228). A pathway implicated by genetic studies in both UC and CD is the IL-23/IL-17 cytokine axis (61). A SNP associated with an amino acid substitution in the cytoplasmic domain of IL-23R conferred significant protection against IBD. Several murine studies also support a pathogenic role for IL-23 in the intestine, ascribed to its ability to drive Th17-mediated inflammation (229–231). In human studies, antibodies against the common p40 subunit of IL-12 and IL-23 led to increased rates of clinical response in CD compared with placebo.

More generally, innate cytokine production is dysregulated in IBD, including TNF and IL-1β (63). This is notably reflected in the efficacy of anti-TNF IgG monoclonal antibodies in the treatment of human disease (156, 232). Furthermore, clinical trials are underway for the use of Anakinra (IL-1Ra), an IL-1β antagonist, in the treatment of severe treatment-resistant UC (IASO trial) (233, 234). In addition to its role in mediating neutrophil inflammatory responses (235), IL-1β has important roles in type 17 immunity in concert with IL-23, particularly in the expansion and maintenance of Th17 cells within inflamed

tissues (236). Strikingly, Ghoresch et al. demonstrated that combined stimulation of naïve T cells with IL-23, IL-1β, and IL-6, in the absence of TGFβ, was sufficient to induce a pathogenic subset of Th17 cells (237), while dual IL-23 and IL-1β stimulation has been shown to bypass the requirement for CD28-mediated co-stimulation for the induction of human Th17 cells (238). Despite the advances in our understanding of how these cytokines drive T cell-mediated pathology in the gut, therapeutic targeting of the downstream effector cytokines has proved unsuccessful, or even detrimental, in the treatment of IBD, as observed with monoclonal antibodies targeting IFNγ or IL-17A (63).

De novo IgG generation has long been associated with chronic intestinal inflammation in IBD patients (9, 10, 98, 239) (**Figure 5**). IBD-associated IgG appears to be directed against components of the commensal microbiome, particularly flagellin (11, 239). A significant proportion of UC patients also develop of auto-antibodies. Perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) are observed in two thirds of UC patients (240–242), while antibodies against colonic goblet cells have been observed in some subjects (243). $CXCR4^+$ IgG plasmablasts have been described in the inflamed colonic mucosa of patients with IBD (200), suggesting local IgG production. However, despite these reports, a systematic characterization of the IBD-associated IgG repertoire and the identification of the cellular mechanisms by which IgG might contribute to IBD pathogenesis has been lacking.

Genetic studies in IBD support the thesis that IgG play a role in UC pathogenesis (**Figure 5**). A variant of FcγRIIA was initially associated with protection from UC in a Japanese case control study (13), and this was confirmed in a metanalysis of GWAS that included data from more than 70,000 patients. The FcγRIIA variant encodes a histidine or arginine at position 131 (H/R131) within the second Ig-like domain, resulting in variable ligand affinity (244, 245), with reduced binding affinity for IgG1 and IgG2 in the R131 variant receptor compared to the H131 receptor (31, 246), the major IgG isotypes detected in the human gut (10). In UC, homozygous R/R131 individuals are protected from disease (odds ratio = 0.63; $P = 1.56 \times 10^{-12}$) (13), suggesting that IgG plays a pathogenic role within the gut rather than being merely a bystander of inflammation. Of note, the FcγRIIA H/R131 SNP has been associated with susceptibility to a number of other autoimmune and inflammatory conditions. For example, the high-affinity H131 polymorphism is associated with Kawasaki disease and systemic vasculitis (247).

To further investigate the role of IgG in UC, we first assessed the extent to the fecal microbes were opsonized by IgA or IgG. In contrast to household controls, where <10% of luminal bacteria were bound by IgG, up to 80% of commensal microbes were IgG-opsonized in UC patients (248). In support of local production of this anti-commensal IgG, we found a marked increase in both heavy chain *Ig* gene transcripts within UC mucosal biopsies, as well as enrichment of several FcγR gene transcripts and associated signaling pathways. To investigate the pathological significant of these observations, we used dextran sodium sulfate (DSS)-induced colitis to interrogate the impact of IgG and FcγR cross-linking of intestinal inflammation. Kobayashi et al. previously demonstrated that exposure to DSS leads to *de novo* production of anti-flagellin IgG, a dominant IgG-targeted antigen in human IBD (249). We confirmed this and demonstrated a more widespread IgG response against multiple bacterial species, including both commensals and pathobionts. This was driven in part by the *de novo* emergence of IgG-producing B cells and plasma cells within the colonic mucosa and GALT, in line with previous observations (9, 98, 200). Immune cell profiling identified $CX3CR1^+$ monocytes and macrophages as the major FcγR-expressing colonic cell types, including expression of the risk receptor FcγRIIA in humans. Furthermore, these cell subsets represent the dominant source of IL-1β production, one of the dominant inflammatory cytokines in human and murine colitis. Passive transfer of anti-flagellin IgG was sufficient to augment pro-IL-1β expression by colonic MNPs in mice exposed to DSS and enhance disease severity in a manner analogous to experiments by Kobayashi et al., whereby naïve mice exposed to anti-flagellin IgG exhibited enhanced weight loss and pathology scores. To assess the contribution of MNP FcγR signaling to this disease augmentation, we made use of transgenic mice with different FcγR A:I ratio through manipulation of the inhibitory receptor, FcγRIIB. $Fcgr2b^{-/-}$ mice exhibited elevated MNP pro-IL-1β expression both *in vitro* and *in vivo*, leading to exacerbated colonic type 17 T cell responses, and impaired recovery from DSS exposure, effects mitigated by IL-1β blockade. In contrast, macrophage-intrinsic FcγRIIB over-expression imparted protection to DSS-induced colitis over non-transgenic littermate controls, demonstrating that MNP-intrinsic FcγR signal strength determines the magnitude of intestinal inflammation.

Our demonstration of the cellular and molecular mechanisms by which IgG-commensal immune complexes might drive inflammation in UC raise several additional questions. Genetic susceptibility studies in IBD to date have focused on *FCGR2A*, but variants in other FcγRs, particularly *FCGR2B*, may be of relevance to disease pathogenesis. For example, FcγRIIB-T232 (rs1050501) results in an isoleucine-to-threonine substitution in the receptor transmembrane domain of the receptor leading to exclusion of the receptor from sphingolipid rafts (250–252). FcγRIIB-T232 is associated with susceptibility to SLE (253, 254), and at a cellular level, with exacerbated pro-inflammatory responses to IgG immune complexes in macrophages and DCs (255). Conversely, the FcγRIIB-T232 genotype is associated with enhanced protection against some infections, including malaria (254). The activating FcγRIIIA-V158 variant, encoding a valine rather than a phenylalanine at position 158, exhibits enhanced affinity for all IgG subclasses (31) and is associated with susceptibility to rheumatoid arthritis (256, 257) and immune-mediated thrombocytopenic purpura. These SNPs in inhibitory FcγRIIB and activating FcγRIIIA have profound effects on IgG-mediated inflammation, and certainly have the potential to influence susceptibility to, or progression of, intestinal inflammatory disease. A comprehensive genetic profiling of *FCGR* polymorphisms in IBD will be required to address this question, but has previously been technically challenging due to the sequence similarity between the FcγR genes (that have arisen by

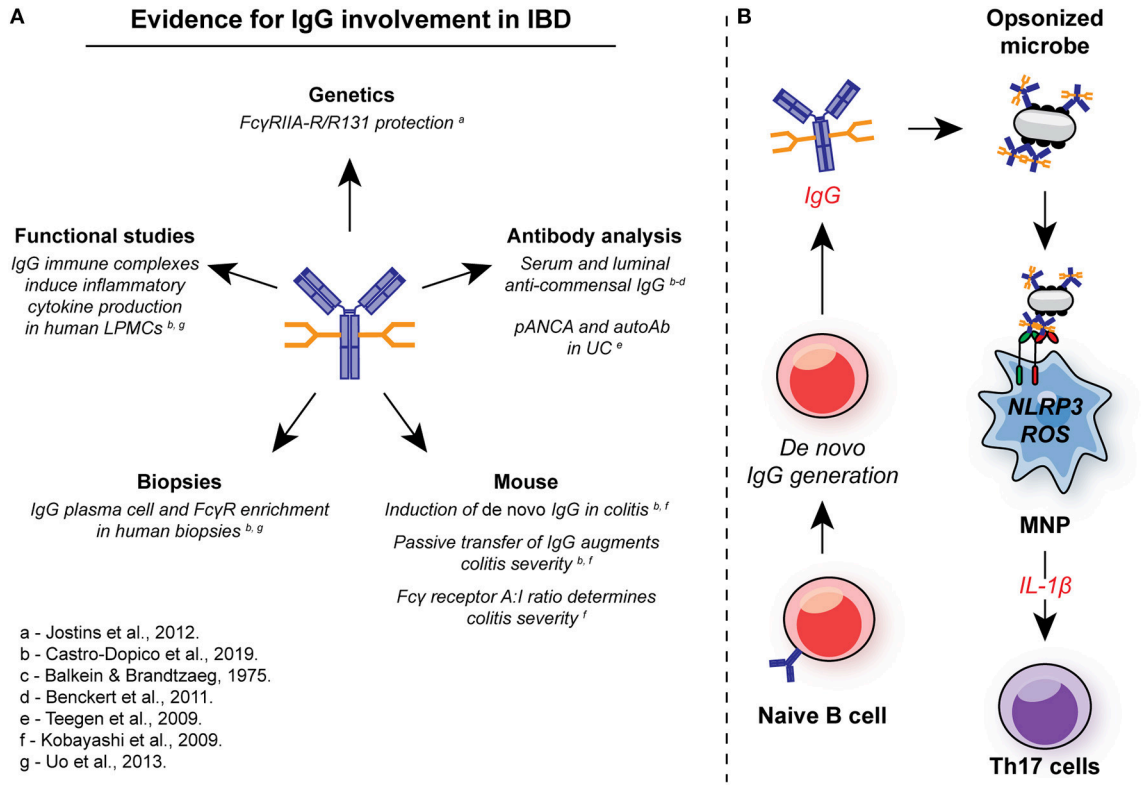

FIGURE 5 | Involvement of IgG in intestinal inflammation. **(A)** There are multiple lines of evidence for the involvement of IgG and FcγR signaling in the pathogenesis in IBD. In genetic studies, the low-affinity FcγRIIA-R131 variant is associated with protection from UC in several independent cohorts. Systemic and local induction of *de novo* anti-commensal IgG and auto-antibodies, such as pANCA, is observed in human IBD, while IgG^+ plasma cells and FcγR-expressing cells are enriched in mucosal biopsies from IBD patients with active disease. In murine studies, *de novo* IgG production is observed, and passive transfer of anti-flagellin IgG to naïve animals exacerbates DSS-induced colitis. FcγR signaling strength determines the magnitude of intestinal inflammation in this model. In humans, IgG immune complex stimulation of intestinal LPMCs drives inflammatory cytokine production, including IL-1β. **(B)** IgG and FcγR in intestinal inflammation: Inflammation is characterized by an increase in the generation of local anti-commensal IgG. These immune complexes cross-link FcγR on colonic MNP, leading to NLRP3 and ROS-dependent IL-1β production. IL-1β, in turn drives Th17 immunity propagating inflammation. ROS, reactive oxygen species; MNP, mononuclear phagocyte; LPMCs, lamina propria mononuclear cells.

gene duplication), and to the copy number variation at this locus (258).

A further interesting question raised by our study is whether abnormalities in the IgG glycome may be present in patients with IBD, and the extent to which these may influence the pathogenicity of anti-commensal IgG. Aberrations in IgG glycome have been described in several autoimmune disorders, and in individuals with impaired responses to infectious agents, such as *Mycobacterium tuberculosis*. The development of widescale IgG profiling via so-called "systems serology" by Alter and colleagues has further implicated distinct IgG glycosylation patterns with IgG functionality and immune reactivity in various disease settings (259–261). Increased agalactosylated IgG has been observed in patients with RA and SLE, a state which favors IgG binding to activating FcγRs, as well as a reduction in IgG sialylation (41, 262, 263). In IBD, abnormal patterns of IgG glycosylation have also been described, with increased agalactosylated IgG in both UC and CD (264), and decreased IgG sialylation detectable in CD (42, 265). Sialylated IgG is associated with increased binding to non-classical Fc receptors, such as DC-SIGN (45), the induction of FcγRIIB on effector cells (122), reduced complement-dependent cytotoxicity (266), and is an essential component of IVIg. Furthermore, agalacotsylated IgG exhibits reduced binding to FcγRIIB (38). Taken together, these data suggest that IgG profiles in IBD are skewed toward a pro-inflammatory phenotype, as observed in other antibody-mediated autoimmune diseases (19). Strikingly, five genes known to regulate IgG glycosylation show robust association with IBD (*IKZF1*, *LAMB1*, *MGAT3*, *IL6ST*, and *BACH2*) (42), including genes encoding galactosyltransferases (41). Given the association between pathological IL-23R signaling and IBD, and the observation that IL-23-derived Th17 immunity promotes IgG class-switching and inflammatory glycosyl patterns (267), these pathways may reinforce one another for the augmentation of pathology in the GI tract.

While the majority of studies focusing on FcγR signaling in GI immunity have focused on MNP and DC biology (200, 217, 248, 268), relatively little is known about how FcγR signaling in neutrophils may contribute to intestinal pathology. Neutrophils are massively expanded in IBD (155) and express FcγRIIA and FcγRIIIB, as well as lower levels of FcγRIIB, making them candidates to directly promote IgG-mediated

inflammation. Furthermore, the emergence of pANCA IgG in two-thirds of UC patients directly implicates neutrophils in the ongoing intestinal humoral response (240–242). Indeed, increased *FCGR3B* gene copy number is associated with susceptibility to UC, directly implicating the FcγR-neutrophil axis in IBD (269). The mechanisms by which this axis leads to disease susceptibility, however, remain unexplored.

IgG in Inflammation-Associated Intestinal Cancer

IBD is associated with a significant risk of developing colorectal cancer. Endogenous and therapeutic monoclonal antibody responses can contribute to tumor rejection *in vivo* through a variety of mechanisms, including DC-mediated T cell activation and NK cell-mediated ADCC (270, 271). Indeed, in the gut, anti-tumor immunity is impaired in the absence of functional IgG responses (268). Consistent with its ability to induce cross-presentation, FcRn-deficient DCs from the MLN of colitogenic mice were impaired in their ability to induce IFNγ production by OT-I cells *in vitro* (149), while MLN CD8 T cells from FcRn-deficient mice were equally impaired in their ability to produce granzyme B and IFNγ following *ex vivo* re-stimulation. In the context of tumorigenesis, DC-specific FcRn expression protected against the development of colorectal cancer and lung metastases in the $Apc^{min/+}$ and DSS/azoxymethane (AOM) models via the homeostatic mucosal activation of endogenous tumor-reactive CD8 T cells (268). This protection was dually dependent on cross-presentation and IgG-IC-driven IL-12 production by DCs. In summary, FcγR-driven immune responses have the potential to contribute to both pathological and protective inflammation in IBD and cancer.

FcγR PATHWAYS IN THE TREATMENT OF INTESTINAL DISEASE

Targeting FcγR Signaling in IBD

Our demonstration of the mechanism by which anti-commensal IgG might drive intestinal inflammation in UC also has therapeutic implications for IBD, particularly given the finding that high levels of colonic IgG and activating FcγR receptor transcripts are associated with resistance to TNF blockade (248). Strategies aimed at reducing the production of pathogenic IgG or blocking its effector function via FcγRs, including B cell depletion, plasmapheresis, and IVIg administration, are commonly used in several inflammatory disorders but their application to IBD has not been studied extensively. No randomized control trials exist for IVIg but a meta-analysis identified a handful of case reports which indicated that IVIg can induce a rapid improvement in steroid resistant CD (272, 273), and there are reports of utility in UC (274). A single randomized controlled trial exists for the anti-CD20 antibody rituximab in UC, where the number of patients included precludes any robust conclusions (275). In UC, of 16 patients who failed to respond to standard therapies, half demonstrated a response at 4 weeks compared to 2 of 8 placebo-treated patients, although this was not maintained to 12 weeks in a further half of patients. However, this study was substantially underpowered, given that studies examining the efficacy of anti-TNF therapies contain hundreds of patients (234). The efficacy of rituximab in depleting mucosal B cells in this study is unclear, given the use of CD20 expression itself to determine depletion, which may be masked by rituximab. Rituximab leads to pan-B cell depletion, which can exacerbate allo- and auto-immunity due to removal of regulatory B cells (276–278) and may be a sub-optimal therapeutic strategy, although rituximab does not appear to exacerbate UC in this instance. Finally, the experimental design of this study does not investigate the effect of repeated B cell depletion, which is associated with long-term treatment response in RA (279). Therefore, the question of whether B cell manipulation may be of benefit in UC has not been adequately addressed.

FcRn inhibitors are currently under development for use in autoimmune diseases, and effectively reduce serum IgG (280–283). Given the prominent role for FcRn in the GI tract, this may also influence IgG epithelial transport and immune complex-mediated local T cell activation within the mucosa of IBD patients.

As well as targeting IgG generation, therapeutic manipulation of FcγR signaling may also prove effective in IBD. Although small molecule SYK inhibitors were shown to be beneficial in RA patients (284), off-target side-effects are common given the widespread expression and function of SYK (285). Modulation of FcγRIIB activity is central to many newer therapies. Enforced co-localization of FcγRIIB with CD19 on B cells using an engineered anti-CD19 monoclonal antibody successfully suppressed humoral immunity in peripheral blood mononuclear cell (PBMC)-engrafted SCID mice (286). Furthermore, small preliminary studies have demonstrated efficacy of soluble human FcγRIIB in the treatment of ITP and SLE (285). However, a deeper understanding of the mechanisms of IgG-mediated inflammation in the GI tract are required for the development of sophisticated therapeutic strategies.

FcγR Influence on Therapeutic Intervention

Beyond targeting IgG-mediated inflammation therapeutically, it is known that FcγR polymorphisms can influence the efficacy of monoclonal antibody therapies in IBD. CD patients homozygous for the high affinity FcγRIIIA-V158 variant demonstrated improved biological responses (as determined by reduced CRP), and a trend toward improved clinical responses, to infliximab compared to FcγRIIIA-F158-bearing individuals (287, 288). NK cells and PBMCs from FcγRIIIA-V158 homozygotes exhibited increased antibody binding and ADCC in response to infliximab. Furthermore, FcγR-dependent effector function has been implicated in mediating protective functions of infliximab at least partly via its ability to form anti-inflammatory immune complexes with trimeric TNF (289, 290). Genetic variation in FcγRIIA can also influence responses to therapeutic monoclonal antibodies, for example the efficacy of rituximab therapy in B-cell lymphomas (291).

FcγR function also has a key role in the effector function of therapeutic monoclonal antibodies in tumor immunotherapy, including checkpoint blockade (292, 293). The specific contributions of FcγR-mediated effector functions have been uncovered through the generation of Fc-optimized antibodies. FcγR-optimized anti-CD25 IgG shows improved ability to

deplete intra-tumoral Tregs by bypassing the upregulation of FcγRIIB by tumor-associated macrophages and cDCs (292). Similarly, anti-CTLA4 IgG requires an Fc domain for activity (294), with hIgG2 antibodies driving FcγRIIA-mediated intra-tumoral Treg depletion in humanized mice (293). Strikingly, FcγRIIIA co-engagement on APCs by anti-CTLA4 IgG also augmented APC-T cell interaction and promoted pro-tumoricidal effector T cell responses (295).

These considerations extend to colorectal cancer, where patients treated with cetuximab, a monoclonal IgG1 antibody directed against EGFR, displayed improved survival in the presence of the high-affinity FcγRIIA-H131 variant (296). However, despite these advancements, a significant proportion of patients remain unresponsive to treatment (293) and further studies are required to determine the impact of these FcγR-mediated effector functions in immunotherapy.

CONCLUSION

Despite the identification of IgG positive cells in colonic biopsies more than 40 years ago by Baklien and Brandtzaeg (9) and the subsequent confirmation of the anti-microbial specificity of this mucosal IgG (239), research into the role of IgG antibodies in an otherwise IgA-dominated organ system has been relatively limited. However, the identification of *FCGR2A*A519G* (rs1801274) as the most-significant non-HLA genetic variant associated with UC in a Japanese GWAS (13), confirmed in candidate gene studies (odds ratio 0.70–0.84) (297, 298) and in a subsequent meta-analysis of IBD GWAS (14), has brought the potential role of mucosal IgG in inflammation into focus.

We and others have shown that microbial IgG forms a core component of the intestinal inflammatory response, both in models of IBD, such as DSS-induced colitis, as well as mucosal infection, potentially identifying novel therapeutic strategies for IBD. However, the role of IgG and FcγRs extend beyond local inflammatory responses and play essential roles in mucosal immune cell education, commensal regulation, oral tolerance, systemic immune protection, and cancer. Future studies will be required to elucidate the signals that determine the generation of these IgG responses in different settings and the mechanisms by which they contribute to local immunity.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Functional Roles of the IgM Fc Receptor in the Immune System

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It is now evident from studies of mice unable to secrete IgM that both non-immune "natural" and antigen-induced "immune" IgM are important for protection against pathogens and for regulation of immune responses to self-antigens. Since identification of its Fc receptor (FcμR) by a functional cloning strategy in 2009, the roles of FcμR in these IgM effector functions have begun to be explored. Unlike Fc receptors for switched Ig isotypes (e.g., FcγRs, FcεRs, FcαR, Fcα/μR, pIgR, FcRn), FcμR is selectively expressed by lymphocytes: B, T, and NK cells in humans and only B cells in mice. FcμR may have dual signaling ability: one through a potential as yet unidentified adaptor protein non-covalently associating with the FcμR ligand-binding chain via a His in transmembrane segment and the other through its own Tyr and Ser residues in the cytoplasmic tail. FcμR binds pentameric and hexameric IgM with a high avidity of ~10 nM in solution, but more efficiently binds IgM when it is attached to a membrane component via its Fab region on the same cell surface (*cis* engagement). Four different laboratories have generated *Fcmr*-ablated mice and eight different groups of investigators have examined the resultant phenotypes. There have been some clear discrepancies reported that appear to be due to factors including differences in the exons of *Fcmr* that were targeted to generate the knockouts. One common feature among these different mutant mice, however, is their propensity to produce autoantibodies of both IgM and IgG isotypes. In this review, we briefly describe recent findings concerning the functions of FcμR in both mice and humans and propose a model for how FcμR plays a regulatory role in B cell tolerance.

Keywords: FcμR, autoantibody, natural IgM, tolerance, Mott cell, epigenetics

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INTRODUCTION

Two forms of IgM exist that differ in the carboxyl terminus of the heavy chain (HC). Alternative splicing with a transmembrane exon (μm) generates monomeric membrane-bound IgM as a B cell receptor (BCR) for antigen and with a secretory exon (μs) polymeric IgM secreted by plasma cell as a component of humoral immunity. The secreted form of IgM consists mainly of J chain-containing pentamers. The existence of J chain-deficient hexamers has also been reported albeit at an unknown concentration. To determine the role of secreted IgM in immune responses, two different groups have independently disrupted the exon encoding the μs (*μs* KO) (1, 2). Such mutant mice normally express IgM and other Ig isotypes on the surface of B cells and secrete all Ig isotypes except for IgM. These mutant mice are unable to control infections, because of inefficient

induction of a protective IgG antibody response (3–5). Paradoxically, the autoimmune pathology associated with IgG autoantibody is more severe in μs KO mice than in the control mice, possibly because of impaired clearance of autoantigen-containing apoptotic cells (6, 7). Yet, no studies have directly demonstrated such deficiency in removal of self-antigens. Thus, both natural and immune IgM are important for protection against pathogens as well as in regulation of immune responses to self-antigens (8).

A variety of secreted and cell surface proteins is involved in binding the Fc portion of antibody, thereby participating in its effector function, e.g., complement and various types of Fc receptors (FcRs). Classical FcRs for switched Ig isotypes (i.e., FcγRs, FcεRI, FcαR), the receptor for polymeric IgA and IgM (pIgR), the low affinity FcεRII/CD23, and the FcR for neonatal IgG (FcRn) have thus far extensively been characterized at both genetic and protein levels (9–17) (see also other articles in this issue), and much of the knowledge gained has now been translated to clinical practice (18, 19). On the other hand, the role of the IgM FcR (FcμR) as an effector molecule for IgM antibody, the first Ig isotype appearing during phylogeny, ontogeny and immune responses, has just begun to be explored, since the *FCMR* was identified in 2009 (20). Several FcμR review articles have recently been published elsewhere (21–25). Here we briefly reiterate the biochemical structure of the FcμR and its functional roles in the development of B cell subsets and plasma cells, describe the potential molecular bases for certain discrepancies observed among different *Fcmr* KO mice, and introduce our theoretical model for how FcμR is involved in B cell tolerance.

UNIQUE PROPERTIES OF FcμR

Dual Signaling Ability

FCMR is a single copy gene located on chromosome 1q32.2 adjacent to two other IgM-binding receptors *PIGR* and *FCAMR* (FcR for IgA and IgM) (20). The predicted human FcμR is a type I glycoprotein of 390 amino acids (aa) with a peptide core of ∼41 kD, which consists of a signal peptide, a V-set Ig-like domain responsible for Fcμ binding, an additional extracellular region with unknown domain structure (termed the stalk region), a transmembrane (TM) segment containing a charged His residue (H^{253}) and a relatively long cytoplasmic (CY) tail of 118 aa containing conserved, three Tyr and five Ser residues (see **Figure 1A**). Among these Tyr residues, the carboxyl terminal Y^{385} matches the Ig tail Tyr motif (DYxN; x indicates any aa) seen in IgG and IgE (26), but the other two do not correspond to any known Tyr-based signaling motifs, ITAM, ITIM or switch. Two carboxyl terminal Y^{366} and Y^{385} are involved in receptor-mediated endocytosis (27, 28) and the membrane proximal Y^{315} is predominantly involved in the FcμR-mediated protection from IgM anti-Fas monoclonal antibody (mAb)-induced apoptosis (28) (see below). An important role of the H^{253} residue in anchoring the receptor in the plasma membrane became evident when the fate of IgM bound to FcμR in cells stably expressing the wild type (WT) or H253F mutant form of receptor was examined by immunofluorescence microscopy; the mutant showed enhanced cap formation even at 4°C. IgM ligand-binding activity was found significantly increased in an FcμR mutant with a deletion of most of the CY tail compared to the WT receptor, despite comparable surface levels as determined by receptor-specific mAbs. Based on our preliminary data, this enhancement appears to result from the formation of an oligomeric FcμR as a consequence of its presumably mobile nature within the plasma membrane. This is different from our speculated inside-out regulation of FcμR ligand binding by its CY tail as seen in integrins. Ligation of FcμR with preformed soluble IgM immune complexes induced phosphorylation of both Tyr and Ser residues (20). Intriguingly, the phosphorylated FcμR migrated faster on SDS-PAGE than the unphosphorylated form, unlike most proteins that run slower when phosphorylated. Preliminary data with an epitope-tagged FcμR suggest that there could be cleavage of the CY tail of FcμR, but the precise molecular mechanisms for this cleavage and the functional role of the resultant FcμR stub still need to be elucidated. Collectively, these features of human FcμR suggest a dual signaling ability of FcμR: one via a potential as yet unidentified adaptor protein non-covalently associating with the FcμR via the H^{253} residue and the other from its own Tyr and Ser residues in the CY tail.

While mouse ortholog with 422 aa has relatively low homology (∼54%) with human FcμR, the overall structural characteristics (a single Ig-like domain, a His residue in TM segment, and a long CY tail containing three Tyr and five Ser residues) are conserved. However, the analysis of its biochemical nature including the ligand binding is limited (22, 29).

Lymphocyte-Restricted Distribution

Given the fact that IgM is the first Ig isotype to appear during phylogeny, ontogeny and immune responses, we initially thought that FcμR would have a broad cellular distribution, thereby serving as a first line of defense against pathogens. On the contrary, FcμR was found to be expressed by lymphocytes only: both B and T cells and, to a lesser extent, NK cells in humans, and only B cells in mice (20, 29–32). Unlike the phylogenetically broad distribution of IgM from jawed vertebrates onward (i.e., cartilaginous fish), computational analysis of existing genomic sequence databases unexpectedly reveals that FcμR appears probably in early reptiles and is found in all three major living (extant) groups of mammals (i.e., egg laying, marsupial and placental mammals) (33). FcμR is the only FcR constitutively expressed on human T cells, which are otherwise generally negative for FcRs, and for B cells, FcμR is the only IgM-binding FcR expressed. [In this regard, another IgM-binding receptor, Fcα/μR, was initially reported to be expressed by B cells, but subsequent analyses revealed that the major cell type expressing Fcα/μR in immune system is a follicular dendritic cell in both humans and mice (34).] During B-lineage differentiation, the cell surface expression of FcμR was detectable from pre-B/B transitional stage to plasmablasts, except for a transient down-modulation during germinal center reactions in both humans and mice (20, 29, 30, 32, 35). Collectively, the restriction of FcμR expression to adaptive immune cells is thus remarkable, because FcRs for switched Ig isotypes are expressed by various hematopoietic cells including myeloid cells as central mediators

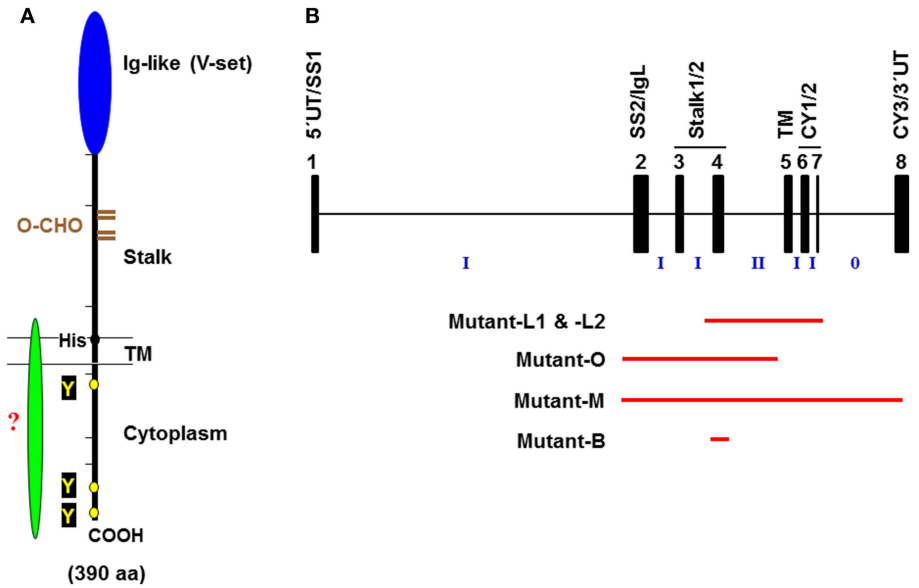

FIGURE 1 | Schematic representation of the FcμR. **(A)** Predicted FcμR protein structure. The human FcμR cDNA encodes a type I transmembrane protein of 390 aa with a peptide core of ∼41 kD that consists of a signal peptide (not shown), an Ig-like domain (V-set), remaining extracellular (stalk), transmembrane (TM; between two lines) and cytoplasmic region. Black and brown hatch marks indicate exon boundaries in the *FCMR* gene and O-glycosylation sites, respectively. Small black and yellow circles indicate a TM charged His residue and conserved Tyr residues, respectively. A green fusiform indicates a hypothetical adaptor protein non-covalently associating with the FcμR ligand-binding chain via the His residue. **(B)** Schematic representation of targeted exons in *Fcmr*-ablated mice. The exon (black closed boxes) organization of *Fcmr* is drawn along with intron phases ("phase 0" indicates between the codons; "phase I" between the first and second nucleotide of a codon; "phase II" between the second and third nucleotide). Exons encoding particular regions of the receptor are denoted as follows: the 5′ untranslated (5′UT), the signal peptide (SS1 and 2), the Ig-like domain (IgL), the uncharacterized extracellular (Stalk 1 and 2), the transmembrane (TM), the cytoplasmic (CY1-3), and the 3′ untranslated (3′UT) regions. Red lines indicate the exons targeted in each *Fcmr* knockout mouse strain (see text for details).

coupling innate and adaptive immune responses. Lymphocyte-specific FcμR may thus have a distinct function from myeloid cell FcRs.

Cis Engagement

Cell surface FcμR in humans is a sialoglycoprotein of ∼60 kD and one third of the relative molecular mass of the mature FcμR is thus made up of O-linked glycans. It exclusively binds the Fc portion of pentameric and hexameric IgM with strikingly high avidity of ∼10 nM as determined by Scatchard plot analysis with the assumption of a 1:1 stoichiometry of FcμR to IgM (20, 25) (**Figure 2A**). Much higher concentrations (>100-fold) are required for binding of monomeric IgM to FcμR-bearing cells, indicating the importance of IgM conformation. This in turn suggests that serum IgM, at its serum concentration of ∼1 μM, constitutively binds to FcμR on the surface of lymphocytes. In addition to the high avidity for IgM in solution, a unique ligand-binding property of FcμR was observed when IgM mAbs to lymphocyte surface proteins were used as a ligand. When Fas death receptor is ligated with 10 pM agonistic IgM anti-Fas mAb, apoptosis-prone Jurkat cells undergo robust apoptosis within 1 day, but Jurkat cells stably expressing FcμR do not (**Figures 2B,C**). This finding is thus consistent with previously reported anti-apoptotic activity of Toso (the original name of FcμR) (36). [In this review we will only use "FcμR" as the name of the receptor, based on a recent nomenclature agreement (37).] However, ligation of Fas with agonistic IgG3 anti-Fas mAb or co-ligation of both Fas and FcμR with the corresponding mouse IgG mAbs plus an appropriate common secondary reagent [e.g., $F(ab')_2$ fragments of anti-mouse γ antibody] had no inhibitory effects on the IgG3 Fas mAb-induced apoptosis (**Figure 2D**). This suggests that FcμR *per se* has no intrinsic activity to inhibit Fas-mediated apoptosis. The anti-apoptotic activity of FcμR depends on usage of the IgM Fas mAb and not on physical proximity of two receptors by artificial co-ligation as observed with ITIM containing receptors such as FcγRIIb and paired Ig-like receptor B (38, 39).

To determine whether the interaction of the Fc portion of IgM Fas mAb with FcμR occurs in *cis* or *trans,* a 10-fold excessive of Fas(−)/FcμR(+) cells as a potential competitive source of FcμR(+) cells was added into the assay but no inhibition of FcμR-mediated protection of Jurkat cells was observed (**Figure 2E**). This suggests that the interaction of the Fc portion of IgM Fas mAb with FcμR occurs in *cis* on the same surface of Jurkat cells (**Figure 2E**), but not in *trans* between neighboring cells (**Figure 2F**). Addition of $>10^4$ molar excess of IgM or its soluble immune complexes was required for partial, but significant blockade of such a *cis* interaction (**Figure 2E**), suggesting that the soluble IgM immune complexes are not potent competitors in the FcμR-mediated protection from IgM Fas mAb-induced apoptosis. However, when IgM mAb reactive with other surface proteins

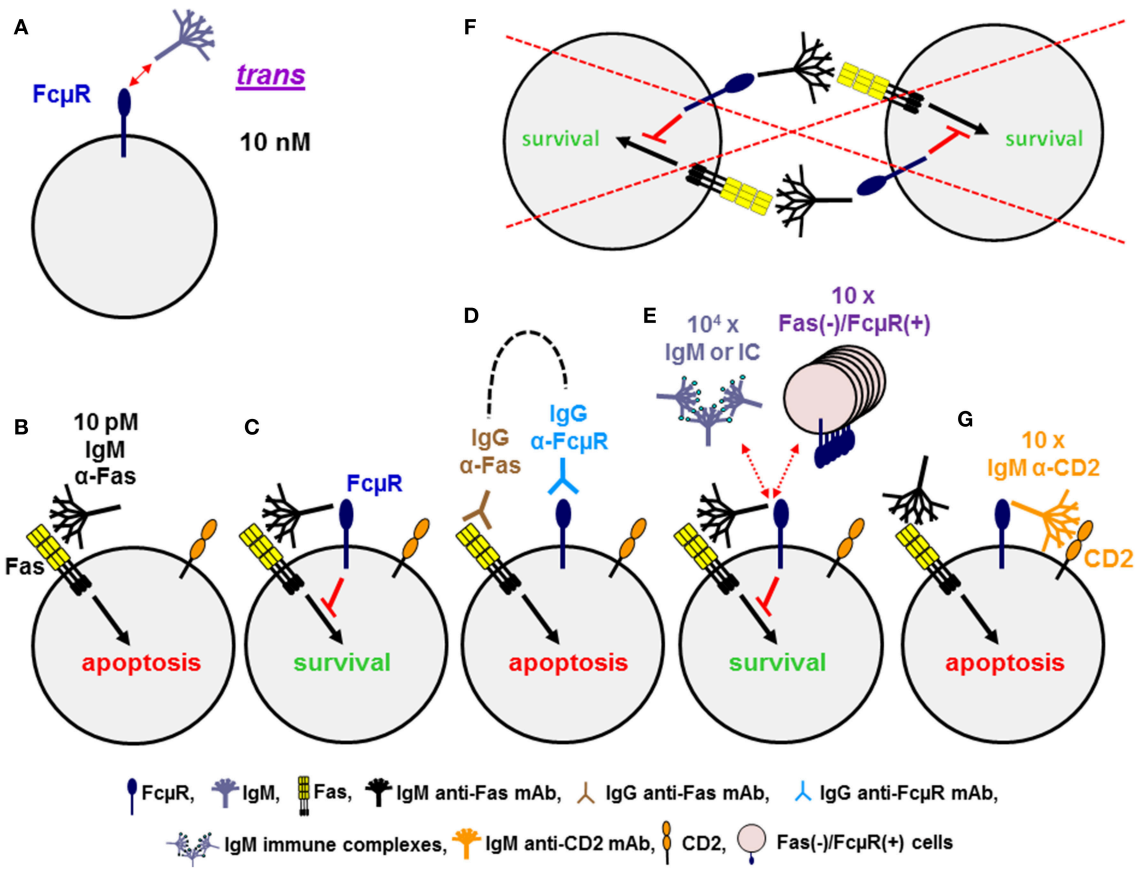

FIGURE 2 | Dominant *cis*, rather than *trans*, interaction of FcμR. **(A)** FcμR positive cells bind IgM pentamers in solution in *trans* with high avidity of ~10 nM. **(B–G)** Ligation of Fas death receptor trimer with agonistic IgM anti-Fas mAb induces apoptosis in WT Jurkat cells **(B)**, but not in FcμR-positive Jurkat cells **(C)**. Co-ligation of Fas and FcμR with the corresponding IgG mAb plus a common secondary reagent (dotted line) has no inhibitory effects on the IgG Fas mAb-induced apoptosis **(D)**. FcμR-mediated protection from IgM Fas mAb-induced apoptosis is not blocked by addition of 10^4 molar excess of IgM or its soluble immune complexes or 10-fold excess of Fas(–)/FcμR(+) cells, suggesting an efficient *cis* interaction of IgM Fas mAb and FcμR on the same cell surface **(E)**, but not a *trans* interaction between neighboring cells **(F)**. Addition of tenfold excess of IgM mAb reactive with CD2 on Jurkat cells can efficiently block the interaction of IgM Fas mAb and FcμR, resulting in apoptosis **(G)**.

expressed on Jurkat cells (e.g., CD2 or TCR) was used as a potential competitor, a 10-fold excess of IgM anti-CD2 or anti-TCR mAb was sufficient to block the *cis* interaction, thereby permitting FcμR(+) cells to undergo apoptosis (**Figure 2G**) (28). Similar results with agonistic IgM vs. IgG3 Fas mAb were observed with Epstein Barr virus-transformed B cell lines simultaneously expressing endogenous FcμR and Fas on the cell surface (20). Furthermore, when BCR and FcμR on blood B cells were co-ligated with a mitogenic IgM anti-κ mAb in the presence of IgG2b anti-FcμR mAbs with blocking or non-blocking activity for IgM-ligand binding, Ca^{2+} mobilization was the same in the absence or presence of FcμR non-blocking mAb. By contrast, the FcμR blocking mAb significantly diminished Ca^{2+} mobilization by blood B cells, suggesting that FcμR provides stimulatory signals upon BCR cross-linkage with IgM mAbs (28). Collectively, these findings of human FcμR indicate that although FcμR binds soluble IgM pentamers and hexamers at a high avidity of ~10 nM, FcμR binds more efficiently to the Fc portion of IgM when it is attached to a membrane component via its Fab region on the same cell surface. FcμR expressed on lymphocytes may thus have a potential to modulate the function of target antigens or receptors when they are recognized by natural or immune IgM through its *cis* engagement.

In summary, FcμR: (i) is expressed by lymphocytes: B, T and NK cells in humans and only B cells in mice, suggesting that FcμR may have a distinct function compared to other FcRs, which are mainly expressed by myeloid cells, and potential species differences; (ii) may have dual signaling ability: one from a potential adaptor protein that non-covalently associates with FcμR ligand-binding chain via H^{253}, and the other from its own Tyr/Ser residues in the CY tail; and (*iii*) binds more efficiently to the Fc portion of IgM when it is attached to a membrane component via the Fab region on the same cell surface (*cis* engagement), than to the Fc portion of IgM in solution/fluids.

VARIANT RESULTS OBSERVED IN DIFFERENT *Fcmr*-DEFICIENT MICE

Despite the initial prediction of embryonic lethality of *Fcmr* ablation (40), there are now five different *Fcmr* KO mice that have been independently generated by four different groups of investigators [Lee et al. (mutant-L1 and -L2), Ohno et al. (mutant-O), Mak et al. (mutant-M), and Baumgarth et al. (mutant-B)]. Eight different groups of investigators have characterized these mutant mice with clear differences in reported phenotypes (29, 32, 35, 41–50) (see **Table 1**). This is an unusual case in the gene-targeting field. Several discrepancies could be in part due to the following: (i) Investigator's preconception of FcμR or Toso in terms of its cellular distribution (B cells vs. myeloid and T cells) (51, 52) and its function (binding IgM Fc vs. inhibiting Fas- or TNFα-mediated apoptosis) (53, 54). (ii) Differences in embryonic stem cells of C57BL/6 (mutant-L1, -L2, and -B) vs. 129/sv (mutant-O and -M) origin and the extent of the 129 mouse-origin DNA still present around the disrupted *Fcmr* gene after backcrossing onto C57BL/6 background; (iii) Differences in exon targeting strategies [exon 2-4 (mutant-O), 2-8 (mutant-M), 4 (mutant-B) vs. 4-7 (mutant-L1 and -L2) (**Figure 1B**)], global (mutant-O and -M) vs. conditional deletion (mutant-L1, -L2, and -B), and the *Cd19* heterozygosity in the CD19-Cre-mediated deletion vs. the unmanipulated *Cd19* in global deletion, and the presence (mutant-M) vs. absence (other mutants) of the *Neo* gene in the mouse genome; and/or (iv) other factors, e.g., ages of the mice examined, experimental procedures/conditions, environmental factors including intestinal microbiota, or reagents used.

Another factor that could contribute to these discrepancies is the relative difficulty in assessing cell surface FcμR in mice by flow cytometry using receptor-specific mAbs, because of its relative low cell surface density as well as its sensitivity to extracellular IgM concentrations, tissue milieu and cellular activation status (20, 29, 35). This vulnerability could result in the discrepancy in reported cellular distribution of FcμR in mice. In fact, using the same receptor-specific rat mAb (B68 clone), FcμR was expressed by: mouse B cells (55), myeloid cells (43) or CD4 T, CD8 T, and B cells (41). This conflicted cellular distribution data about FcμR is a major reason why some investigators created additional Cre/loxP-mediated, cell type-specific *Fcmr* deletion systems (35, 50). In this regard, EIIa-Cre mediated *Fcmr*-deleted mutant-L1 (equivalent to global deletion) showed more TNFα-induced apoptosis of CD3/CD28-activated CD8 T cells than control mice (41). The abnormality was initially considered as an intrinsic T cell defect since this group originally reported that FcμR was expressed by T cells. However, subsequent results from conditional deletion clearly indicated no phenotypic differences between T cell-specific [or dendritic cell (DC)-specific] *Fcmr* deletion and control counterparts. The only differences were seen with B cell-specific *Fcmr* deletion. The authors thus concluded that FcμR on B cells might indirectly affect certain T cell functions (50), although it remains unclear how this would work. In this review, we will focus on the following aspects of B cell-related findings in *Fcmr* KO mice: (i) alterations in B cell subsets, (ii) IgM homeostasis, and (iii) dysregulated humoral immune responses.

Alteration in B Cell Subsets

The development of B-lineage cells in the bone marrow (BM) was unaffected in most *Fcmr* KO strains (29, 32, 35, 50) except for mutant-M where the numbers of pro-B, pre-B, and immature B cells were significantly diminished as compared to WT controls (42). Since the surface expression of FcμR begins to be detectable at the transitional stage of pre-B to B cells in differentiation, it seems conceivable that FcμR is dispensable in developing B-lineage cells in the BM. However, it is noteworthy that: (i) μs KO mice, which are deficient for secretion of IgM, have significantly altered B cell development from pre-B to the immature B cell transition (42); (ii) this alteration of early B cell development is corrected by administration of natural IgM (56); and (iii) many of the abnormalities observed in *Fcmr* KO mice mirror those seen in μs KO mice. Thus, despite the fact that FcμR is a key sensor of secreted IgM, it remains to be elucidated why, among five *Fcmr* KO mice, only mutant-M has an alteration in development of B cell precursors (42). In this regard, several human pre-B cell lines express FcμR transcripts but not FcμR protein on their cell surface at detectable levels unless stimulated with phorbol myrystate acetate (20, 57), suggesting the existence of post-transcriptional controls of FcμR.

Unlike in the BM, in peripheral lymphoid organs there were variable alterations in B cell subsets observed in these mutant mice but, as a general trend, *Fcmr* ablation was found to more profoundly affect innate-like B cells, B-1, and marginal zone (MZ) B cells, rather than the B-2 or follicular (FO) B cell compartment (see **Table 1**). Remarkably, an increase in B-1 B cell numbers, particularly in spleen accompanied by elevated levels of autoantibodies of both IgM and IgG isotypes, has been the sole result consistently observed in all five mutant mice. Thus, FcμR plays an important regulatory role in the homeostasis of B-1 B cell development and autoantibody production (see further discussion below).

For MZ B cells, the mutant-O had age-dependent alterations in their cell numbers, i.e., increase in young (3-wk) and marked decrease in old (>9-wk) mice (49). This age-dependent reduction of MZ B cells might result from their rapid differentiation into plasma cells in the absence of FcμR, as evidenced by the markedly elevated IgM autoantibodies to Smith antigen/ribonuclear protein, which are considered to be derived from MZ B cells (45). Alternatively, FcμR-deficient MZ B cells might undergo cell death due to lack of survival signals through FcμR upon BCR cross-linkage (49), as shown by cross-talk downstream of FcμR and BCR signaling via the non-canonical NFκB pathway (47). Notably, the reduction of MZ B cells was also observed with both *Fas*- and *Fcmr*-deficient, autoimmune-prone B6.MRL.$Fas^{lpr/lpr}$/$Fcmr^{-/-}$ mice (45). In mutant-M, unlike mutant-O, there were no changes in the MZ B cell compartment, whereas in both CD19-Cre-mediated deletion mutant-B and -L2, the number of MZ B cells was not reduced (for mutant-B) or enhanced (for mutant-L2) (35, 50). Since the number of MZ B cells in μs KO mice is increased by 3-fold and this increase can be normalized by passive administration of natural and polyclonal,

TABLE 1 | Phenotypic comparison of five different *Fcmr*-deficient mice.

***Fcmr* KO created by**	**Lee et al. (mutant-L1)**	**Ohno et al. (mutant-O)**					**Mak et al. (mutant-M)**					**Baumgarth et al. (mutant-B)**	**Lee et al. (mutant-L2)**
Δ exons; ES cell origin	**4–7; C57BL/6**	**2–4; 129/Sv**					**2–8; 129/Sv**					**4; C57BL/6**	**4-7; C57BL/6**
***Neo* cond. KO**	**Removed Cond. KO (EIIa-Cre)**	**Removed Global del. (backcrossing with C57BL/6)**					**Not removed Global del. (backcrossing with C57BL/6)**					**Removed cond. KO (CD19-Cre)**	**Removed cond. KO (CD19-, CD4- or CD11c-Cre)**
References (year)	(41) (2011)	(29) (2012)	(45) (2014)*	(32) (2012)	(47) (2015)	(49) (2018)	(42) (2013)	(43) (2013)	(44) (2014)	(46) (2015)	(48) (2015)	(35) (2017)	(50) (2018)
Distribution of FcμR+ cells	B68 mAb: CD4 T, CD8 T, NKT, B & Leuko. in Sp, LN, blood	MM3 mAb: B cells; immat. B to plasma-blast in BM, Sp, LN	*NR*	4B5 mAb: B cells	4B5 mAb: B cells	MM3 mAb: B cells	mRNA: B, Gr-1+, CD11c+ in Sp	B68 mAb: granulo. & mono. in BM, Sp	*NR*	*NR*	*NR*	4B5 mAb: B cells; TGN in immat. B in BM	B68 mA: B cells
FcμR levels & function in WT mice	α-CD3/CD28 Rx of CD8 T: ↑	Sp: FOB > MZB > NFB; B1 = B2 PerC: B1a = B2 > B1b		BM: immat. B >proB/preB Sp: MZB > FOB PP: non-GCB > GCB	α-μ Rx of B: ↑; co-IP of FcμR/IgM BCR; α-μ & α-FcμR Rx: ↑p52,↑BCL-xL		BM: immat. B > pro-B, pre-B; Sp: FOB > MZB > GCB, PC; PerC: B2 > B1a;					BM: mat. B > immat. B > late preB; Sp: MZB > B1 > FOB;	BM: mat. B > immat. B, preB
Lymphocyte populations in BM	*NR*	Not changed	CD19+ cells↓	Not changed	*NR*	Not changed	cells↓, proB↓, preB↓, immat. B↓, mat.B→	*NR*	*NR*	*NR*	*NR*	B1, B1a, B1b→	Not changed
Lymphocyte populations in periphery	Sp, LN and Blood: B↓, CD8 T→ , CD4 T→	Sp: B→ , FOB→ , MZB↓, B1 ↑; LN: B→ , T→ ; PerC: T↑	Sp: B↓, B2↓, B1a & 1b → , MZB↓, PC↓	Sp:B→ , FOB→ , MZB↓, Tr3B↓, $IgM^{hi}IgD^{hi}$↑	*NR*	Sp: MZB↑ & FOB↑ at 3wk; MZB↓ at 9 wk	Sp: cells↓, FOB↓; PerC: B1a↑, B2↓	*NR*	*NR*	*NR*	*NR*	Sp: B1↑, B1a↑, FOB↑, MZB→ ; PerC: B1→ , B1a→ , B1b→ ; splenomegaly (8 mo)	Sp: TrB↑, mat. B→ , B1↑, B2→ ,FOB→ , MZB↑; PerC: B1a↑, B1b↓
Effect of *Fcmr*-ablation	↑Suscept. of act. CD8 T to TNFα-induced apoptosis Resistance to TNF/GalN-induced, iNKT-mediated liver damage	↑Basal serum IgM & IgG3 ↑Nat. autoAb of IgM, IgG3 & IgG2c ↑Ab resp. to TI-2 Ag (subopt. dose) ↓1° IgG1 & ↓2° IgM Ab resp. to TD-Ag	↑Basal serum IgM & IgA at early age ↑Nat. autoAb of IgM & IgG ↑MZB-derived IgM α-Sm/RNP Ab Rapid PC- diff. of MZB cells ↑Mott cell formation	↑Basal serum IgM ↑Nat. autoAb of IgM & IgG ↓Ab resp. to TI & TD Ags ↓Survival after BCR ligation ↓GC formation ↓Memory & PC-diff.	↓Survival of B cells upon α-μ Rx → BCR-mediated endocyto → plκBα & ↓BCL-xL after α-μ Rx	↑Cell death & turnover of MZB cells ↑IgD and MHC II on MZB cells ↓tonic BCR signaling ↓Ab resp. to TI-1 Ag ↑Suscept. to sepsis	Basal serum ↓IgG1 (3 mo), ↑IgG3 & IgA (6 mo) ↑nat. IgG autoAb Ab resp. to: TI-1↑, TI-2→ , TD 1° IgG↓ & 2° IgG→ ↓prolif. & survival after α-μ Rx	→ Myeloid development ↑ROS after fMLP Rx of granulocytes ↓phagocyte *Listeria* ↓TNFα, IL-6 after LPS Rx resistance to endotoxin shock *in vivo* suscept. to *Listeria* inf.	Resistant to MOG-EAE ↓T & Mϕ infiltration in brain → CD4 T function ↓DC act. & maturation ↑Treg diff.	↓Recruit. and act. of iDC in LCMV-liver ↓act. of CD8T in liver fail to induce autoimmune diabetes	↓IL17A, ↓IL10 & ↑INFγ in Th17-polarizing cells	↑IgM BCR ↑tonic BCR signaling ↑basal serum IgM ↑ASC of IgM & IgG in Sp, BM ↑nat. autoAb of IgM & IgG ↑survival of unstimulated B1 & B2 cells.	↑Suscept. to H1N1 infection in CD19-Cre mice only → Ag-specific CD4 & CD8T resp. ↓TNFα & INFγ by CD4 & CD8 T ↓B cell survival ↑IL10+ Breg → basal serum IgM & IgG ↑natural autoAb of IgM &IgG

**Mutant-O mice crossed with Fas-deficient autoimmune prone B6/lpr strain.*

α-, anti-; Ab, antibody; act., activation; Ag, antigen; ASC, antibody secreting cell; BCR, B cell receptor; BM, bone marrow, cond., conditional; del., deletion; diff., differentiation; FOB, follicular B cells; GCB, germinal center B; iDC, inflammatory dendritic cell; immat., immature; inf., infection; iNKT, invariant NKT; KO, knockout; LN, lymph node; MZB, marginal zone B; nat., natural; NFB, newly formed B; NR, not reported; PC, plasma cell; PerC, peritoneal cavity; PP, Peyer's patch; prolif., proliferation; ROS, reactive oxygen species; Rx, treatment; recruit., recruitment; Sp., spleen; subopt., suboptimal; suscept., susceptibility; Sm/RNP, Smith antigen/ribonuclear protein; TD, T cell dependent; TGN, trans-Golgi network; TI, T cell independent; Tr(3), transitional (3); WT, wildtype; 1°, primary; 2°, secondary; ↑, ↓ & → , increased or enhanced, decreased or diminished, & comparable to WT control mice.

but not monoclonal, IgM, we initially considered that the FcμR and its signals upon IgM-ligand binding might play an important regulatory role in the fate of MZ B cells. This simplistic view, however, may need further consideration based on the above conflicting results.

In addition to the aforementioned changes in cell numbers of B cell subsets, there were some differences in the density of certain cell surface markers (e.g., CD19, CD21, CD23, IgD, IgM) between *Fcmr* KO and WT controls (29, 49, 50). The surface levels of several co-receptors of the BCR complex such as CD21 and CD23 were diminished in certain B cell subsets from mutant-O compared to WT controls (29, 50). This was also the case with CD19 that was also significantly diminished on BM immature B cells, but not on BM recirculating and splenic B cells in mutant-O (our unpublished observation). Notably, the surface IgD levels were higher on splenic MZ B in mutant-O mice than WT controls (49). Indeed, our subsequent analysis of the same mutant mice also revealed higher expression of surface IgD on BM recirculating and splenic MZ B cells, but not on FO B cells, than WT controls (unpublished). The molecular basis for this elevated surface IgD density in mutant-O is unclear, but it has been shown that functionally hypo-responsive anergic B cells are characterized by high levels of IgD BCR and generally turn over rapidly when competing, non-tolerant B cells are present (58, 59).

For surface IgM in mutant-O, IgM staining with fluorochrome-labeled anti-μ mAb, which might include endogenous membrane-bound IgM plus cytophilic IgM bound via FcμR or other potential IgM-binding proteins/receptors, was indistinguishable in these B cell populations including BM immature B cells (29, 49). By contrast, in mutant-B the cell surface expression of IgM BCR was significantly increased as compared to control mice, but this phenotype was only demonstrable 3 days after transferring of *Fcmr*-deficient or control B cells into *μs* KO mice to avoid the influence of cytophilic IgM (35). The authors implied that this increase in IgM BCR in *Fcmr* KO mutant-B was due to the lack of FcμR-mediated constraints on the IgM BCR (see below), resulting in enhanced tonic BCR signaling, facilitating the spontaneous differentiation of B-1 B cells and the increase in autoantibody production. Stimulated emission-depletion microscopic analysis revealed a strong interaction of FcμR with membrane-bound IgM in the *trans*-Golgi network (TGN) of BM immature B cells, but a weak interaction with the IgM on the plasma membrane in mature B cells, thereby constraining transport of IgM to the plasma membrane. This effect on the exocytotic pathway was proposed to regulate surface expression of IgM and eventually limiting tonic IgM BCR signaling. When we examined the potential interaction of FcμR with IgM BCR on the plasma membrane by fluorescence resonance energy transfer, we also found a very low incidence of such an interaction. By contrast, another group showed the physical interaction of FcμR and IgM BCR on the plasma membrane of mature B cells by confocal microscopy (47) and that tonic BCR signaling was diminished in *Fcmr* KO mutant-O (49). Given the low avidity of FcμR for monomeric IgM in solution, it remains unclear how FcμR could interact with membrane-bound IgM in the TGN of BM immature B cells or on the plasma membrane of mature B cells.

Another remarkable finding related to this issue came from immunofluorescence confocal microscopic analysis: strong staining of intracellular FcμR in a region corresponding to the TGN in murine BM immature B cells (35). The results were in close agreement with the findings of FcμR-mediated endocytosis of IgM by chronic lymphocytic leukemia (CLL) B cells in humans (27). The bulk of the intracellular FcμR protein resided in the TGN and in small vesicles, probably sorting endosomes of CLL cells. While the major function of the TGN is to sort proteins destined for the plasma membrane, endosomal compartment or specialized secretory granules, retrograde transport in the endocytic route to the TGN has been demonstrated for several proteins (60). It is thus worth considering whether DNA- or RNA-containing autoantigens are engulfed into endosomes by IgM BCR on immature B cells in the BM, two thirds of which are known to be autoreactive at least in humans, followed by retrograde transport to the TGN where TLR9 or TLR7 recognizes the respective DNA or RNA/IgM BCR complexes and then FcμR binds the Cμ3/Cμ4 of the resultant oligomerized IgM BCRs in the TGN.

IgM Homeostasis

The pre-immune serum level of IgM or natural IgM was elevated in most *Fcmr* KO mice (29, 32, 35, 50) except for mutant-M (42) and this elevation correlated with the number of *Fcmr* null mutant alleles ($Fcmr^{-/-} > Fcmr^{+/-} > Fcmr^{+/+}$) (32). The frequency of IgM-secreting cells in spleen and BM was significantly higher and the spot sizes in ELISPOT assays were also bigger in mutant-B than their control counterparts (35). FcμR was not expressed by phagocytic cells in spleen and liver including liver sinusoidal endothelial cells, which are thought to be the primary site of IgM catabolism at least in rat, as determined by both immuno-histological and RT-PCR analyses (29). The half-life of injected IgM was comparable between *Fcmr* KO (mutant-O) and WT mice. Thus, the increase in serum IgM levels in naive *Fcmr* KO mice is the consequence of lack of FcμR-mediated regulation of natural IgM production either at the B cell or plasmablast stage in innate-like B cells (29).

Dysregulated Humoral Immune Responses

Antibody responses to T cell-independent (TI) and T cell-dependent (TD) antigens were dysregulated in *Fcmr* KO mice as compared to WT controls, although there were some differences among mutant mice that might result from differences in mouse ages, antigen doses and forms, administration routes, kinetics, etc. Generally, mutant mice exhibited enhanced TI type 2 responses (involving multiple BCR cross-linkage) but impaired TD responses, especially at suboptimal doses. Since similar selective enhancement of TI-2 immune responses are also observed in *μs* KO mice (2) and mice deficient for components of the BCR complex such as CD19 (61) or CD81 (62), FcμR seems to regulate B cell responses to TI-2 and TD antigens by interacting differently with BCR complexes on the plasma membrane.

In summary, there are clear differences in reported phenotypes in five different *Fcmr* KO mice in terms of development of B cell subsets and plasma cells, IgM homeostasis and humoral immune responses. However, the increase in

B-1 B cell compartment accompanied by elevated levels of autoantibodies of both IgM and IgG isotypes is the sole result consistently observed with all these mutant mice.

EPIGENETIC FINDINGS IN THE *Fcmr-Il10* LOCUS IN TREG CELLS

One of the biggest discrepancies in the field is the cellular distribution of FcμR in mice (B cells vs. non-B cells). While several groups of investigators described the predicted functions of FcμR in non-B cell populations, their actual evidence for the surface expression of FcμR by myeloid, dendritic and T cells was rather weak (41, 43, 51, 52). Most of their functional results came from the comparative analysis in chimeras adoptively transferred by a mixture of *Fcmr* KO and WT BM cells or the direct comparison of cellular function between *Fcmr* KO and WT controls (43, 44, 46, 48). This was the reason why the phrase "functional relevant expression of FcμR" by non-B cells was used (52). Nevertheless, several functional outcomes in non-B cells from some *Fcmr* KO mice could be worthy of consideration because of the clear-cut differences compared to WT controls, even though they might be indirect or bystander effects. For example, mutant-M were resistant to the induction of myelin oligodendrocyte glycoprotein (MOG)-induced autoimmune encephalomyelitis (EAE). The authors initially considered that this resistance was not due to an intrinsic impairment of mutant Th1 and Th17 cell functions (see different observations by another group of investigators below), but rather to the immature and tolerogenic nature of mutant DCs, as characterized by their weak inflammatory responses and increased induction of Treg cells (44). Intriguingly, administration of a recombinant soluble FcμR fusion protein, which consisted of the human FcμR ectodomain and human IgG1 Fc (lacking complement binding activity) (FcμR EC/IgG Fc), into EAE-susceptible WT mice resulted in delaying or ameliorating their disease, depending on the time points of injection. While its mode of action was not discussed, it might be possible that since IgM anti-MOG antibody also participates in the demyelination process in EAE, the soluble FcμR EC/IgG Fc could simply act as a decoy receptor.

By contrast, results from recent single-cell RNA sequencing analysis along with complex algorithmic assessments and its functional annotation indicated that FcμR is one of the four critical regulators of Th17 pathogenicity in MOG-induced EAE (48). [The other three included *Gpr65* (G protein-coupled receptor 65), *Plzp* (promyelocytic leukemia zinc finger transcriptional repressor of the Th2 master regulator *Gata3*) and *Cd5l* (CD5-like antigen, apoptosis inhibitor expressed by macrophages [AIM], or soluble protein α [Spα]). Astonishingly, CD5L/AIM/Spα is a glycoprotein of ∼45 kD secreted by macrophages, supports their survival and was originally identified as an IgM binding protein (63–65). Two out of four regulators identified for Th17-mediated EAE were thus capable of binding to IgM, although CD5L/AIM/Spα was annotated as a regulator of lipid biosynthesis (66).] Th17 cells polarized *ex vivo* by differentiation conditions with TGFß+IL-6 or IL-1ß+IL-23+IL-6 from *Fcmr* KO mutant-M were found to secrete significantly less IL-17A and IL-10 than those from control WT mice (48). Mutant naive CD4 T cells exhibited lower FOXP3 levels during Treg cell differentiation upon TGFß stimulation *in vitro*. The authors considered that FcμR could be a negative regulator in a non-pathologic state but a promoter of pathogenicity (48), although it was difficult to understand its mechanisms. Given our findings that none of the sorted T cells with the phenotype of IL-17$^+$, INFγ$^+$, or IL-17$^+$/IFNγ$^+$ expressed FcμR transcripts, as determined by gene array analysis (25), it is hard to imagine how such a minor population of Th17 cells expresses functional FcμR, possibly at low levels, on their surface and plays a major regulatory role in the pathogenesis of EAE.

To explore the molecular basis for the resistance of *Fcmr* KO mice to EAE as well as for the reduction of IL-10 production by their Th17 cells, a computational epigenetic analysis was performed. Since *Fcmr* and *Il0* genes are ∼139 kb apart from each other on chromosome 1, we analyzed the data of the histone post-translational modification by chromatin immunoprecipitation and sequencing and the assay for transposonase-accessible chromatin (ATAC) sequencing available for resting and activated Treg cells at the *Fcmr-Il10* locus (67). These included marks of acetylation of histone H3 at lysine 27 (H3K27ac) as a predictor of enhancer activity (68, 69), albeit not exclusively, and of ATAC as an indication of open chromatin (70). As shown in **Figure 3**, the H3K27ac marks are selectively observed in three loci, i.e., 3′ site of *Fcmr*, 5′ upstream of *Il10* and *Il10,* in activated Treg cells. The ATAC and H3K27ac marks coincided, suggesting that these loci were in an opened chromatin status, hence transcription factors would be highly accessible to these loci. Remarkably, the H3K27ac marks in the *Fcmr* gene of activated Treg cells were restricted to its 3′ region, i.e., exon 5 (TM) to exon 8 (encoding CY tail and 3′ UTR) and were absent in exon 2 (encoding the Ig-like domain responsible for IgM-ligand binding), consistent with the lack of functional FcμR expression by T cells. This 3′ *Fcmr*-restricted H3K27ac mark was not observed with resting Treg cells, suggesting that the potential enhancer activity of 3′ *Fcmr* in Treg cells was dependent on cell activation. By contrast, the H3K27ac marks in the 5′ upstream of *Il10* were observed irrespective of cell activation. Notably, several regions besides exons in the 3′ *Fcmr* were conserved in 40 other placental mammalian *Fcmr* genes as determined by phastCons (not shown). The above H3K27ac marks were not observed in early B-lineage cells, i.e., pro-B cells of either young or old mice. Collectively, these three loci [3′ site of *Fcmr*, 5′ upstream of *Il10*, and *Il10*] could be involved in enhancing IL-10 expression by Treg cells upon cellular activation potentially through a chromatin loop formation.

While the above epigenetic results of the *Fcmr-Il10* locus were derived from Treg cells, it remained to be elucidated whether a similar scenario was applicable for other cell types including Th17 cells. If so, *Fcmr* KO mutant-M, in which exons 2–8 were targeted, do not have this putative 3' *Fcmr* enhancer element for IL-10 in their genome, and this could account for the reduction of IL-10 production by Th17-polarizing cells (48). For *Il17a*,

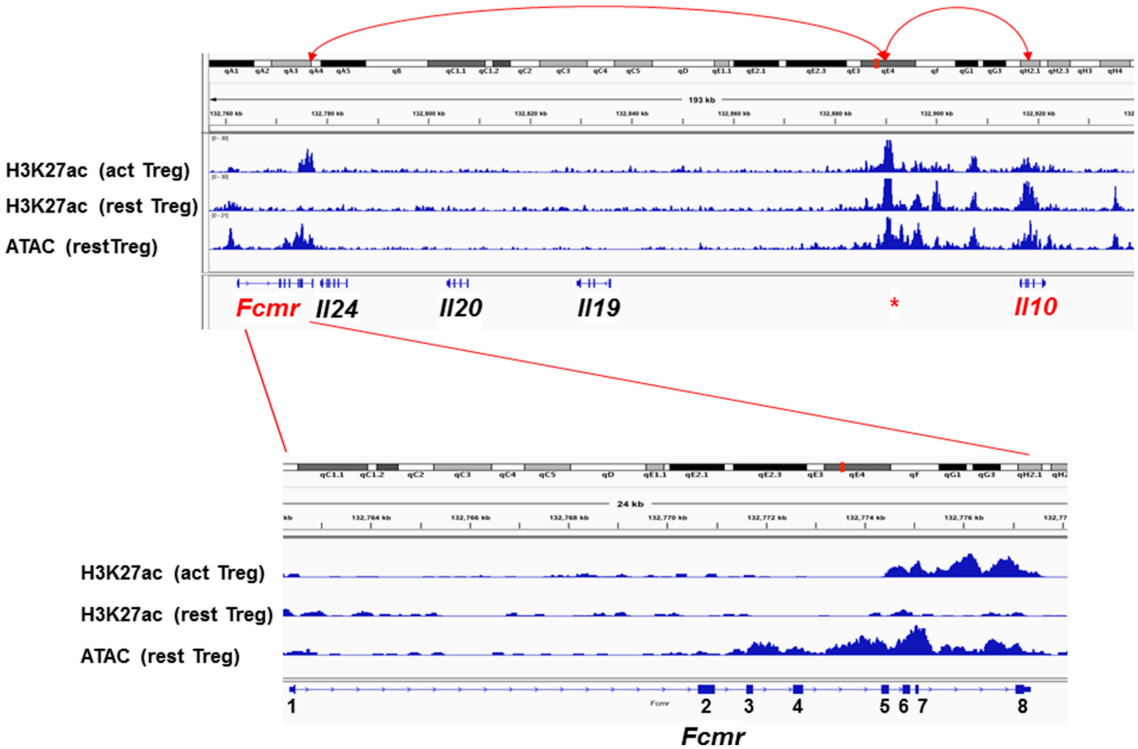

FIGURE 3 | Epigenetic status of the *Fcmr-Il10* locus in Treg cells. Top: Genomic locus (~180 kb) from *Fcmr* to *Il10* is depicted along with the chromosome 1 site designation, distance, marks of the acetylation of histone H3 at lysine 27 (H3K27ac) in activated (act) or resting (rest) Treg cells, marks of the assay for transposonase-accessible chromatin (ATAC) and the exon (square) and intron (line) of indicated genes. Red * indicates the unique region with high H3K27ac marks in both activated and resting Treg cells and of ATAC at 5′ upstream of *Il10* gene. Red arrow lines indicate potential association with the indicated loci by chromatin loop formation. Bottom: Enlarged illustration of *Fcmr* locus with coding exons numbered. 5′ and 3′ UTR regions are indicated by smaller squares.

which is located at ~110 Mb upstream of *Fcmr* on chromosome 1, whether the 3′ *Fcmr* enhancer element is able to form such a long-range interaction with the *Il17a* promoter is an intriguing question. It is also unclear how absence of the 3′ *Fcmr* enhancer element contributes to the resistance to EAE in *Fcmr* KO mutant-M. Nevertheless, given the assumptions that in single-cell RNA sequencing analysis, most identified FcμR transcripts might be derived from its 3′ region and that only the resistance to EAE as the consequence of *Fcmr*-deficiency might be functionally annotated for FcμR, it is thus conceivable and very intriguing that FcμR could be one of the four important regulators of Th17 pathogenicity in EAE, despite the lack of expression of functional FcμR by such T cells (48). Collectively, some of the discrepancies observed in *Fcmr* KO mice could be attributed to differences in the exons disrupted.

In summary, the epigenetic analysis of *Fcmr-Il10* locus reveals that three loci (3′ site of *Fcmr,* 5′ upstream of *Il10,* and *Il10*) may be involved in enhancing IL-10 expression by Treg cells upon cellular activation through chromatin loop formation. The epigenetic alteration selectively at the 3′ site of *Fcmr* may account for the functional abnormalities in non-B cell populations observed in certain *Fcmr* KO mice in conjunction with the exons targeted, even though functional FcμR is not expressed by such non-B cell populations.

FcμR IN CENTRAL DELETION OF AUTOREACTIVE B CELLS DEVELOPING IN BONE MARROW

The common feature among the different *Fcmr* KO mice is the propensity to produce autoantibodies of both IgM and IgG isotypes accompanied by increases in B-1 B cells, indicating an important regulatory role of FcμR in B cell development and central repertoire selection against those B cells expressing autoreactive BCRs. During B cell development in the BM, immature B cells are highly susceptible to deletion by BCR crosslinking. It has been estimated that ~90% of the newly generated BM B cells are deleted before entering the mature B cell compartment (71) and that approximately two thirds of the BM immature B cells in humans are self-reactive (72). During this development, the FcμR expression becomes detectable at the transition from BCR-non-expressing pre-B cells to BCR-expressing immature B cells. In three strains of mutant-O, -B and -L2 (29, 32, 35, 50), however, the sizes of the pro-, pre- and immature B cell compartments showed no alterations, when compared with WT control mice. Only one mutant-M had reduced pro-, pre-, and immature B cell compartments (42). Changes in sizes of BM B-lineage compartment might not become visible in such analyses, because such changes in the

number of BCR^+ B cells might occur, as the immature B cells exit the BM. Furthermore, the peripheral compartments of immature and mature B cells may fill by homeostasis to unaltered sizes, though with either non-autoreactive or autoreactive B cells.

It is noteworthy that *μs* KO mice, which are deficient for secreted pentameric IgM, the ligand of FcμR, have significantly altered B cell development at the transition from pre-B to immature B cells (42). This alteration of early B cell development, including the inability to centrally delete autoreactive B cells, can be corrected by administration of natural IgM (56). Therefore, ligation of the FcμR by its ligand, pentameric natural, polyclonal IgM *in vivo* contributes to the negative selection of autoreactive B cells. It remains to be elucidated in this experimental setting whether immature B cells in BM are the prime target of this correction. If so, it suggests that the provision of pentameric, natural, polyclonal IgM binding to FcμR on immature B cells allows *cis*-crosslinking of autoreactive BCRs with autoantigen presented by pentameric IgM ligated to FcμR (**Figure 4**). This crosslinking would be expected to connect signaling from the BCR (e.g., via PI3 kinase) (73) with signaling from FcμR. If FcμR-signaling would downregulate PI3 kinase activity, this could lead to upregulation of FOXO1, which, in turn, could upregulate RAG1/2 expression (74, 75). In this way the immature B cells could continue editing VL-JL-rearranged light chain (LC) gene loci (76, 77) to change the autoreactivity of the BCR. Any loss of autoreactivity would abolish *cis*-crosslinking with autoantigen-bound natural IgM/FcμR, thus terminate RAG expression and allow immature B cells to leave the BM.

FcμR AND MOTT CELL FORMATION IN THE CONTROL OF AUTOIMMUNITY OF B CELLS

Another finding is the marked increase in Mott cells in mutant-O, even though it has only been described by our analysis (45). We propose that the FcμR may control autoantibody production by formation of Mott cells in the scenario described below. Mott cells are a variant form of plasma cells containing Ig inclusion bodies (called Russell bodies) that accumulate in dilated rough endoplasmic reticulum (ER). Mott cells are rarely observed in normal lymphoid tissues, but are found in various pathological conditions, such as Ig-associated neoplasms, chronic inflammatory diseases and autoimmune disorders (78–81). Several mechanisms for formation of Ig inclusions or for the defect in Ig secretion have been suggested, including (i) structural alteration of Ig HCs preventing their appropriate processing, (ii) impairment of Ig LCs in preventing Ig HC aggregation, and (iii) inability to degrade or to export Ig, leading to its aggregation. However, the most relevant mechanism associated with mutant-O seems to be that the Ig becomes stuck in the exocytotic pathway due to its autoreactivity with intracellular membrane components. Several precedents support this idea. (i) Two clonally unrelated IgM Mott cell hybridomas utilize germline Ig variable gene segments and have no obvious structural defects, suggesting their B-1 B cell origin (79). (ii) Ig inclusions are not generated when the Mott Ig μ HC or κ LC is by itself or is associated with a heterologous κ LC or μ HC, respectively. The inclusion body formation is only reconstituted when Mott VH and Mott Vκ genes are expressed with an IgM, but not IgG1, constant region, suggesting that both specificity and isotype are critical for Mott cell formation. (iii) LPS or IL-5 stimulation of sorted B-1 B cells from autoimmune mice (NZB/W F_1) generates Mott cells *ex vivo* at a frequency of ∼50 times higher than conventional B-2 B cells (81). (iv) In studies of autoantibody transgenic mice, incompletely edited B cells express multi-reactive IgM that accumulates in the Golgi and is released or detached from the membrane as insoluble amyloid-like immune complexes termed spherons reaching up to ∼2 μm in diameter (82, 83).

Given these precedents and the preferential *cis* engagement of FcμR, the following scenario would account for the high incidence of Mott cells in the absence of FcμR. Incompletely edited B cells migrate into peripheral lymphoid tissues and express membrane-bound IgM with self-reactivity to intracellular membrane components (e.g., glycans). The interaction of the monomeric IgM with self-antigens in the ER must be of low affinity. However, when cells receive certain signals such as from TLR4 to facilitate a switch in the usage of μm to μs exon along with J-chain synthesis during transition to the ER-Golgi intermediate compartment (ERGIC) or the Golgi, the resultant pentameric IgM is contained inside the ERGIC/Golgi vesicles and binds a self-antigen on intracellular membranes via its Fab region and simultaneously the FcμR via its Fc portion. This *cis* engagement may prevent further differentiation of such autoreactive B cells, thereby contributing to the peripheral tolerance to self-antigens located on intracellular membranes (**Figure 4**). Based on this hypothesis, Mott cell IgMs in *Fcmr* KO mice are anticipated to have autoantibody activity to intracellular membrane components.

Instead of IgM-opsonized self-antigens, it may be equally possible that DNA and DNA-associated autoantigens or RNA and RNA-associated autoantigens are recognized by the respective IgM on BM immature B cells and delivered to an endosomal or lysosomal compartment where TLR9 or TLR7 binds the corresponding ligand-containing IgM BCR. The resultant oligomerized IgM BCRs are transported via a retrograde route to the TGN where FcμR may bind the Cμ3/Cμ4 domain of the oligomeric IgM BCR. In summary, based on the findings of enhanced autoantibody production in all *Fcmr* KO mice and Mott cell formation in our mutant mice as well as the *cis* engagement of FcμR, we propose a model for how FcμR on B cells plays a regulatory role in central and peripheral tolerance.

FcμR IN DISEASES

The association of FcμR with human CLL has long been suggested, dating back to studies showing that CLL B cells could form rosettes with ox erythrocytes coated with IgM antibody (84, 85). By flow cytometric assays CLL B cells also exhibited specific IgM binding (57, 86). Subsequently, several investigators showed enhanced *TOSO/FCMR* gene expression in CLL and initially considered that this enhancement would

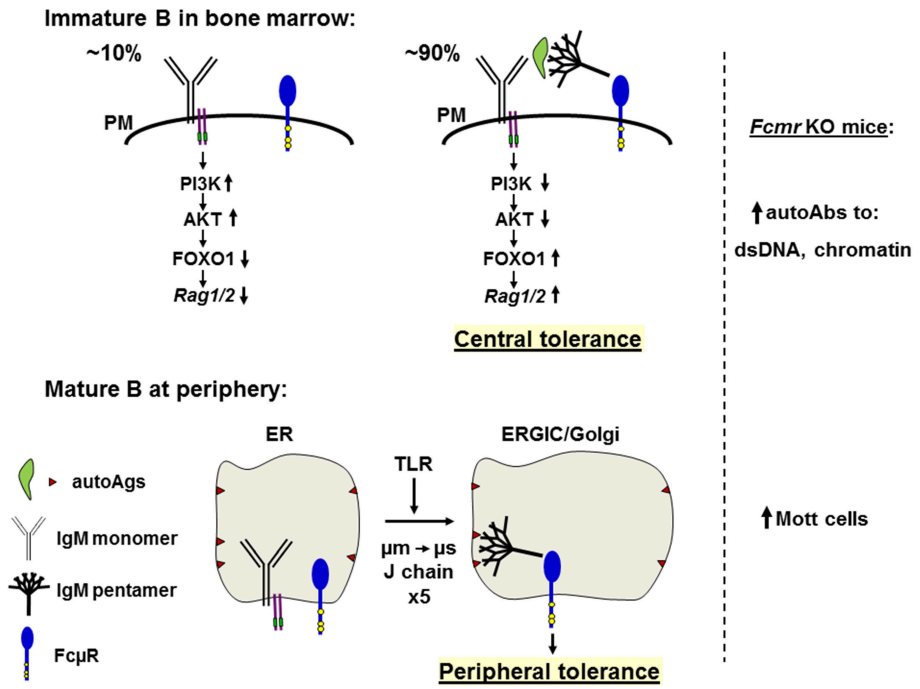

FIGURE 4 | Hypothetical model of the role of FcμR in central deletion and B cell repertoire selection. Top: In the bone marrow, only small populations (~10%) of the newly generated B cells bearing monomeric IgM (Y shape with Igα/Igβ, two purple lines carrying green ITAM) on the plasma membrane (PM) are survived by ligand-independent (or tonic) signals through activation of the PI3K-AKT pathway and suppression of the FOXO1-mediated Rag1/2 activity (left). By contrast, ~90% of the newly generated immature B cells have variable binding affinities for autoantigens (green leaf shape) and are subjected to negative selection by receptor editing or apoptosis (right). Autoantigens opsonized with natural pentameric IgM (broom shape) simultaneously bind the corresponding BCR and FcμR (blue tennis racket shape with three conserved Tyr residues in yellow). The resultant cross-linkage of BCR and FcμR on immature autoreactive B cells may inhibit the BCR-mediated PI3K-AKT pathway, resulting in relief of the AKT-mediated suppression of FOXO1 and leading to activation of Rag1/2 and receptor editing, thereby contributing to negative selection or central tolerance. In the absence of FcμR autoantibodies against dsDNA or chromatin are increased. Bottom: In peripheral lymphoid tissues, incompletely edited B cells express IgM BCR with self-reactivity to membrane components (brown triangles) present on ER membranes, but no interaction of monomeric IgM with the corresponding antigens occurs due to its low affinity. When cells receive signals from TLR, a switch from μm to μs exon usage occurs along with the synthesis of J chain during the translocation from ER to the ER Golgi intermediate compartment (ERGIC) or the Golgi and the resultant pentameric IgM is contained inside the vesicles where it binds membrane components via the Fab regions and its Fc portion binds FcμR. This *cis* engagement of self-antigen/secreted IgM/FcμR within the vesicles prevents further development of such autoreactive B cells, thereby contributing to peripheral tolerance. In the absence of FcμR, Mott cells containing intracellular Ig inclusion bodies are increased.

contribute to increased resistance of CLL cells to apoptosis (87, 88). We also examined the surface expression of FcμR by B and T cells in CLL using receptor specific mAbs by flow cytometry. CLL B cells ($CD19^+/CD5^+$) expressed significantly much higher levels of surface FcμR than B cells from healthy donors. This enhancement was more evident in Ig HC variable region (*IGHV*)-mutated, better prognostic, $CD38^-$ or early Rai-stage CLL than in *IGHV*-unmutated, poor prognostic, $CD38^+$ or advanced Rai-stage CLL (89). Intriguingly, surface FcμR levels were also significantly elevated in non-CLL B cells ($CD19^+/CD5^-$) and T cells ($CD19^-/CD5^+$), especially in patients with *IGHV*-mutated CLL, when compared with the corresponding populations in healthy individuals. This increase in FcμR expression on T cells in CLL was unique, because normal human T cells activated *ex vivo* with anti-CD3 mAb or PMA down-modulated surface FcμR, whereas normal B cells activated with anti-μ mAb or PMA up-regulated surface FcμR (20). Regarding the enhanced surface expression of FcμR on CLL B cells, CLL-derived BCRs, unlike those from other B cell malignancies, have been shown to ligate each other via interactions between Ig HC CDR3 of one BCR and the framework region 2 of another BCR irrespective of their *IGHV* mutation status, thereby providing antigen-independent cell-autonomous signaling (90, 91). This antigen-independent self-ligation of BCR on CLL cells could account for enhanced surface expression of FcμR as well as for the well-known phenomenon of reduced levels of surface IgM and IgD on CLL cells. It remains unclear, however, why surface FcμR levels were also elevated on non-CLL B and T cells in *IGHV*-mutated CLL patients.

Another remarkable finding was the marked elevation of serum titers of FcμR in CLL patients but not in healthy individuals (89). [One exception was an individual who was found 2 years later to have high serum autoantibody titers against dsDNA.] Detection of the serum FcμR was accomplished by sandwich ELISA using two different receptor-specific mAbs. It was resolved as an ~40 kD protein, distinct from the ~60 kD cell surface FcμR and found by proteomic analysis as a soluble form

of the receptor (solFcμR), which was encoded by an alternative spliced FcμR transcript resulting from the direct splicing of exon 4 (stalk 2) to exon 6 (CY1), skipping exon 5 (TM). This splicing event resulted in a reading frame shift in exon 6 and generated a novel 70 aa hydrophilic carboxyl tail, thereby confirming the source of the solFcμR. The functional role of solFcμR in CLL and possibly in autoimmune disorders as observed with aforementioned exceptional control individual remains to be elucidated. In this regard it is noteworthy that administration of another form of solFcμR (FcμR EC/IgG Fc) into EAE-susceptible mice ameliorates the disease (44). Collectively, both membrane-bound and soluble forms of FcμR are elevated in patients with CLL.

Since among leukemia/lymphomas CLL uniquely expresses high levels of FcμR on their surface, two types of immunotherapy targeting for the receptor have thus been developed for CLL cells. One is an immunotoxin-coupled IgM Fc (Cμ2-Cμ4) and the other is chimeric antigen receptor-modified T cells using a single chain fragment-containing the variable region of an anti-FcμR mAb (6B10) (92, 93). In both cases, patient CLL B cells appear to be selectively eliminated *in vitro* without affecting the non-leukemic B and T cells. Apart from FcμR in hematologic malignancy, *FCMR*-deficiency has not yet been identified, but based on the data from *Fcmr* KO mice it may belong to hyper-IgM syndrome. Since FcμR is expressed by B, T, and NK cells in humans, the phenotypic abnormalities of *FCMR* deficiency in affected individuals are predicted to be more complex than those in *Fcmr* KO mice. In patients with selective IgM immunodeficiency, we initially predicted that surface FcμR levels might be high because of lack of ligand-induced down-modulation. Contrary to this assumption, cell surface FcμR levels on a particular circulating B cell subset with a MZ phenotype ($IgM^+/IgD^+/CD27^+$) in such patients were significantly diminished as compared to age-matched controls, but the molecular basis for this reduction remains to be elucidated (94).

In summary, enhanced levels of both the membrane-bound and secretory forms of FcμR are evident in patients with CLL, possibly as the consequence of antigen-independent autonomous self-ligation of BCR on CLL cells.

EPILOGUE

It has been known for many years that passive administration of IgM antibody enhances the subsequent antibody responses to antigenic challenge, whereas passive administration of IgG antibody suppresses the response. Complement activation, but not its lytic activity, has so far been implicated as a mechanism for this IgM-mediated enhancement, and the inhibitory FcγR is involved in IgG-mediated suppression (95, 96). The existence of FcμR on a variety of cell types has also been suggested for nearly 50 years by many investigators including us, but the FcμR cDNA was identified just 10 years ago by a functional cloning strategy (20). However, since FcμR turned out to be identical to the Toso cDNA, which was also previously cloned by functional strategy as a potent inhibitor of Fas-mediated apoptosis, there have been lively debates regarding the real function of this receptor, IgM Fc binding vs. Fas-apoptosis inhibition. While we have now a general consensus that this is an authentic FcμR, there have been clear discrepancies in the phenotypic abnormalities reported in five different *Fcmr* KO mice. In this article, we have discussed potential molecular mechanisms underlying some of these discrepancies. One of the remarkable outcomes of our analysis is the finding of restricted H3K27ac and ATAC marks to the 3' *Fcmr* in activated, but not resting, Treg cells and could account for some puzzles in T cell function described in certain *Fcmr* KO mice. Given the fact that all *Fcmr* KO mice are prone to produce autoantibodies accompanied by increased B-1 B cells, we introduce our hypothetical model for how FcμR controls autoantibody production. We see that FcμR has a very important role in immature B cell in the BM to control against the development of autoreactivity in B cell repertoire. We hope that this short article may help to resolve many still existing puzzles and will open new avenues of investigation.

AUTHOR CONTRIBUTIONS

PKJ performed the comparative analysis (**Table 1**). KH analyzed the FcμR ligand binding property (**Figure 2**) and the phenotype of mutant-O. NO and SS conducted the epigenetic analysis (**Figure 3**). AR intellectually contributed. HK and FM made the rest of figures (**Figures 1**, **4**) and wrote the paper. All authors listed approved for publication. PKJ was a scholar of the Alexander von Humboldt Foundation.

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REVIEW
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The Role of Antibodies and Their Receptors in Protection Against Ordered Protein Assembly in Neurodegeneration

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Ordered assemblies of proteins are found in the postmortem brains of sufferers of several neurodegenerative diseases. The cytoplasmic microtubule associated protein tau and alpha-synuclein (αS) are found in an assembled state in Alzheimer's disease and Parkinson's disease, respectively. An accumulating body of evidence suggests a "prion-like" mechanism of spread of these assemblies through the diseased brain. Under this hypothesis, assembled variants of these proteins promote the conversion of native proteins to the assembled state. This likely inflicts pathology on cells of the brain through a toxic gain-of-function mechanism. Experiments in animal models of tau and αS pathology have demonstrated that the passive transfer of anti-tau or anti-αS antibodies induces a reduction in the levels of assembled proteins. This is further accompanied by improvements in neurological function and preservation of brain volume. Immunotherapy is therefore considered one of the brightest hopes as a therapeutic avenue in an area currently without disease-modifying therapy. Following a series of disappointing clinical trials targeting beta-amyloid, a peptide that accumulates in the extracellular spaces of the AD brain, attention is turning to active and passive immunotherapies that target tau and αS. However, there are several remaining uncertainties concerning the mechanism by which antibodies afford protection against self-propagating protein conformations. This review will discuss current understanding of how antibodies and their receptors can be brought to bear on proteins involved in neurodegeneration. Parallels will be made to antibody-mediated protection against classical viral infections. Common mechanisms that may contribute to protection against self-propagating protein conformations include blocking the entry of protein "seeds" to cells, clearance of immune complexes by microglia, and the intracellular protein degradation pathway initiated by cytoplasmic antibodies via the Fc receptor TRIM21. As with anti-viral immunity, protective mechanisms may be accompanied by the activation of immune signaling pathways and we will discuss the suitability of such activation in the neurological setting.

Keywords: prion-like proteins, neurodegeneration, tau (MAPT), Fc receptor, microglia, antibody immunity, alpha-synuclein, beta-amyloid

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PROTEOPATHY IN NEURODEGENERATION

Following the death of his patient, Auguste Deter, in 1906, Alois Alzheimer described the presence of abundant extracellular plaques and intracellular neurofibrillary tangles in her brain (1). These lesions were subsequently shown to be widely distributed in the brains of sufferers of the disease that went on to take Alzheimer's name. The plaques and tangles are now known to comprise of assemblies of the proteins amyloid-β (Aβ) and hyperphosphorylated microtubule associated tau, respectively. Alzheimer's disease (AD) is the most common of a heterogeneous family of age-related neurodegenerative disorders characterized by the deposition of specific protein assemblies in the brain. This includes progressive supranuclear palsy (PSP), corticobasal degeneration and Pick's disease, where tau deposition is observed; dementia with Lewy bodies and Parkinson's disease (PD) where cytoplasmic protein α-synuclein (αS) deposits are observed; sporadic Creutzfeldt-Jakob disease, where the membrane-anchored prion protein, PrP, is deposited and, finally, amyotrophic lateral sclerosis where TAR DNA binding protein 43 (TDP-43) is implicated. The common characteristics of the protein assemblies among these pathological conditions is that they exhibit an ordered fibrillar structure, known as amyloid, as well as a range of smaller assemblies generally referred to as oligomers. Together, the age-related neurodegenerative diseases are one of the most pressing biomedical and societal problems. Dementia, of which AD is the most common cause, affects around 50 million people worldwide and numbers are expected to double before the middle of the 21st century. Critically, there are currently no treatments that slow or prevent the progression of any of the age-related neurodegenerative diseases.

Findings over the past few decades place protein aggregation as a central mediator of pathology. Human genetics has revealed numerous mutations in the genes that encode the aggregating proteins themselves. A suite of more than 40 mutations in tau cause inherited dementias, with evidence of tau fibrils in brain tissue (2). Mutations in αS lead to inherited forms of Parkinson's disease and, in certain cases, an acceleration of *in vitro* αS fibrilization (3, 4). Mutations in the gene that encodes amyloid precursor protein (APP), the protein from which the Aβ peptide is derived, lead to increased levels of the aggregation-prone $A\beta_{42}$ and familial AD (5). Other mutations in genes responsible for processing these proteins, such as the proteases responsible for the generation of Aβ, or in clearing misfolded proteins species, such as the AAA ATPase p97/VCP, can also lead to inherited variants of neurodegenerative diseases (6, 7). Collectively, these genetic associations suggest that the accumulation of protein aggregates causes neurodegeneration. For AD, the prevailing framework of disease progression is the amyloid cascade hypothesis (8, 9). Under this hypothesis, the accumulation of Aβ plaques drives pathological consequences that include the formation of tau fibrils and neuronal cell death. Therapeutic approaches in AD have therefore focused on preventing the production of Aβ, or promoting its clearance. A series of disappointing, high profile clinical trials have led to the critical reappraisal and amendment of the amyloid cascade hypothesis, or to propose earlier intervention, since the downstream events unleashed by Aβ accumulation may be irreversible (5, 10). Therapeutic approaches that target tau in AD are therefore considered promising routes for future intervention. Of the 20 therapeutic strategies that target tau that have reached clinical, nine are based on passive transfer or eliciting of antibodies (11). A further two therapies that target αS have also reached the clinic. Immunotherapy therefore represents one of the brightest hopes for modifying disease progression in age-related dementias.

PROTEIN ASSEMBLIES AS PROPAGATING ENDO-PATHOGENS

The occurrence of protein deposits was long considered a cell-autonomous feature of neurodegeneration. Over the past few decades, this view has been challenged by a body of research demonstrating that pathological protein conformations can provoke native protein to adopt the assembled form. By consuming pools of native cellular proteins, the assembled variants can sustain their propagation through time and space within the affected brain. The prototypic example of this behavior is the prion protein, PrP, wherein the normal cellular variant, PrP^{C}, is converted to a pathogenic variant, PrP^{Sc}. Most cases of prion disease are sporadic or inherited though, in rare cases, disease can be acquired from the environment by eating diseased meat or human brain as occurred in now-abandoned tribal rituals. The model of templated protein aggregation was proposed as a common mechanism in neurodegeneration when it was shown that Aβ could be induced to aggregate in mice expressing APP (12, 13). As an extracellular peptide, the seeded aggregation of Aβ likely relies on direct contact between introduced seed and the available pools of peptide. For other proteins such as tau and αS, which are expressed in the cytoplasm, pools of native protein are maintained within cell-limiting membranes, thereby limiting contact between seed and substrate. Seeded aggregation of cytoplasmic proteins was nonetheless demonstrated when AD brain homogenate was found to induce tau pathology in mice expressing wild-type human tau (14). In cultured cell systems, protein misfolding could be transmitted from the extracellular environment to cytoplasmic tau pools (15). Similar properties have been demonstrated for αS, TDP-43, and huntingtin, the protein whose expanded polyglutamine tract is implicated in Huntington's disease (16–19). Thus, although diverse in their clinical manifestations, it is possible that age-related neurodegenerative diseases share a common "prion-like" mechanism of dissemination though affected brains (20) (**Figure 1**).

Understanding the molecular mechanisms governing the transfer of pathology between cells is central to any mechanism-based intervention. For immunotherapy against tau and αS, the issue is key as it determines which pool of protein should preferentially be targeted. *In vitro* studies in neurons demonstrate that tau misfolding can be transferred across synapses (21, 22). This is consistent with animal work, which suggests that tau pathology is preferentially transmitted between

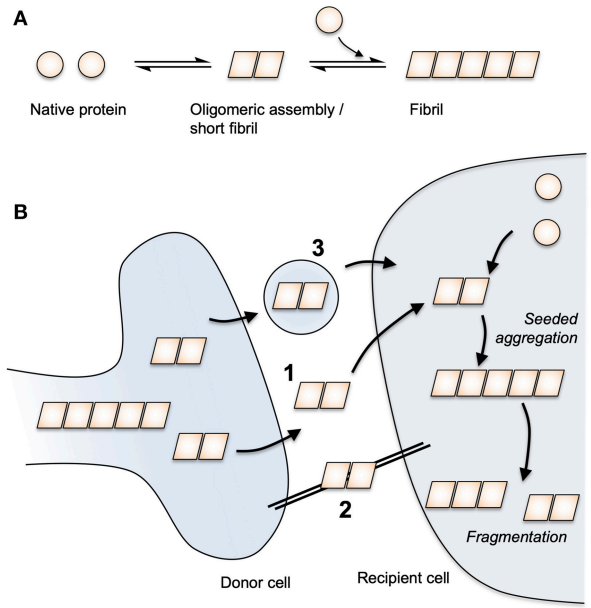

FIGURE 1 | Protein aggregation and prion-like spread. **(A)** Native protein undergoes a spontaneous conversion to an assembled state. Assemblies above a critical size are able to extend via the addition of native protein monomers to form a fibril. **(B)** Assembled protein species are able to transmit between cells via routes that may include (1) free protein release and uptake, (2) tunneling nanotubes and (3) extracellular vesicles. Once taken up to the cytoplasm of a neighbouring cell, seeded aggregation occurs through the templated addition of native protein. By fragmenting, fibrils can exponentially amplify in number.

connected regions of the brain (23–25). Furthermore, imaging of human brains using positron emission tomography (PET) tracers reveals that network connectivity is correlated with tau pathology, consistent with transfer of tau misfolding along synaptically connected pathways in the brain (26). Extracellular naked protein assemblies transiting between neurons thus represent an attractive target for immunotherapy as they are physically accessible to antibodies. However, other mechanisms of intercellular transfer have been described. For instance, exosomes and extracellular vesicles can contain tau and transmit pathology (27, 28) and tunneling nanotubes, actin-containing structures that bridge cells, can transmit pathology in culture (29, 30). There also remains discussion around the contribution of prion-like spread vs. cell-autonomous aggregation (31). Assuming that protein seeds are not obtained from the environment, then cell-autonomous aggregation must, at least, be responsible for the generation of the original seed. In this way, the extent to which pathology is governed by cell autonomous vs. prion-like mechanisms, and the route of such spread, define the limits of what any given therapeutic approach can achieve.

ANTIBODIES IN THE BRAIN

IgG levels are maintained in human serum at around 10 mg/ml. The brain is isolated from serum by the blood-brain barrier (BBB), which is impermeable to large macromolecules including IgG (32). The brain, instead, is bathed in cerebrospinal fluid (CSF), which is produced following the filtration of blood and transport of ions across the choroid plexus. The resulting concentration of IgG in CSF is around 500- to 1,000-fold lower than in serum. At face value, this low concentration of antibody in the brain makes CNS antigens unattractive as targets for passive immunotherapy, which is normally administered to the periphery. This is compounded by a poor understanding of the mechanisms by which steady state levels of antibody are maintained. CSF flows around the brain, before exiting the CNS along spinal and cranial nerves and via drainage to the lymphatic system (33, 34). Intrathecally administered IgG is rapidly cleared from the brain, largely through this bulk flow and with a possible contribution of selective transport out of the brain. The neonatal Fc receptor, FcRn, is expressed in abundance at the BBB (35). Given FcRn's role in transcytosis of antibodies across the placenta, it has been suggested that FcRn may perform reverse transcytosis to help maintain the low IgG environment of the CNS. There is some evidence that antibody clearance from the brain is mediated in part by the antibody Fc domain (36, 37), and export of an anti-Aβ monoclonal antibody was reduced in an FcRn-deficient mouse (38). However, the brain concentration of peripherally administered IgG was not significantly different between wild-type mice and mice lacking FcRn (39). This speaks to a need for further investigation of how antibody levels in the CNS are maintained, with a particular requirement to understand the rate of transit across the BBB (**Figure 2**). Under a model where antibodies are maintained at static, low levels in the CNS, there is little scope for achieving meaningful binding occupancy to intracerebral antigens. However, if there exists a rapid cycling of antibodies in and out of the brain, total exposure of antibodies to antigen will, over time, be substantially greater. Evidence in support of a high flux dynamic equilibrium comes from experiments that measured the rate of clearance of intrathecally administered IgG, which demonstrated a half-life of <1 h in a primate model (40). This compares to a half-life of around 3 weeks in the periphery. Thus, peripherally produced or administered antibodies, particularly if they have high affinity for their antigens, may gain sufficient exposure to meaningfully engage intracerebral antigens.

The assertion that antibodies can bind antigens in the brain and exert biological effects is supported by several strands of clinical evidence. For instance, there is a growing set of neuroimmune diseases associated with auto-antibodies that bind to neuronal targets (41, 42). Antibodies against the membrane-associated protein amphiphysin are causally linked to the rare progressive disease stiff-person syndrome (43). Antibodies against antigens such as Zic4 and Yo/PCA1 are associated with cerebellar ataxia and antibodies against N-methyl-D-aspartate receptor (NMDAR) are commonly associated with encephalitis (44). From animal and clinical studies it has been shown that peripherally administered antibodies that target Aβ can engage their targets and induce reductions in brain amyloid load (45–49). Though an unwanted side effect, the ability of passive immunotherapy against Aβ to induce lesions (amyloid-related imaging abnormalities or ARIAs, discussed further below), stands as further testament to ability of antibodies

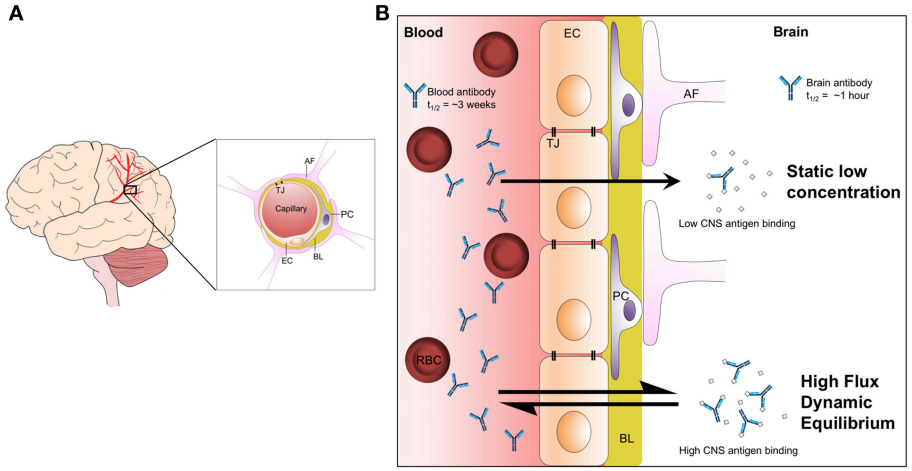

FIGURE 2 | The maintenance of brain antibody levels. **(A)** Graphical representation of blood vessels in the brain and the cellular structure of the BBB. Endothelial cells in blood vessels interact via tight junctions, restricting the passage of solutes to the CNS. Pericytes bind to the basal lamina and provide structural support to the barrier. Astrocytic foot processes extend from the interstitial spaces to interact with the basal lamina and surrounding cells. **(B)** Two models of antibody cycling into the CNS. Under a model of static, low concentration in the CNS, antigen binding is highly restricted. However, a model where antibodies rapidly cycle in and out of the brain permits continuous bathing of brain antigens in dilute antibody solution. Over time, this model allows much higher levels of antigen binding. Evidence in support of such a model includes the observation that antibody half-life in the brain is <1 h, compared to around 3 weeks in serum. AF, astrocyte foot; BL, basal lamina; EC, endothelial cell; PC, pericyte; RBC, red blood cell; TJ, tight junction.

to engage targets in the brain parenchyma. Together, these clinical observations stand as strong evidence that antibodies in circulation can penetrate the brain and engage their targets at a level sufficient to exert biologically relevant effects.

FC RECEPTORS AND THEIR EXPRESSION IN THE BRAIN

FcγRs are expressed on the surface of a wide range of immune effector cell types and bind to the Fc region of IgG. The canonical FcγRs are divided into those that activate immune signaling upon binding to antibody (in humans these are FcγRI, FcγRIIa, FcγRIIc, and FcγRIIIa), one that exerts inhibitory function (FcγRIIb), and one neutral glycosylphosphatidylinositol (GPI)-linked receptor, FcγRIIIa, which lacks cytoplasmic domains and is highly expressed on neutrophils (**Table 1**). There are four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) with varying affinity for the different receptors. The high-affinity interactions are between FcγRI and all IgG subclasses except IgG2, and between FcγRIIIa and IgG3 (50). The high-affinity interactions permit binding to free IgG molecules, yet are not of such high affinity that they preclude responses to IgG-labeled multivalent complexes (50). There is a widespread, but erroneous, belief that IgG4 is a neutral subclass of IgG. In fact, it binds all FcγRs, albeit with slightly lower affinity than IgG1 (50, 51). However, IgG4 does not fix complement and can inhibit IgG1-mediated complement fixation (52, 53). Uniquely among the human antibody subclasses, IgG4 undergoes arm exchange, resulting in chimeric, bispecific antibodies (54). In mice, the FcR system is broadly similar, with activating FcγRs (FcγRI, FcγRIII, and FcγRIV) and one with inhibitory activity (FcγRII) (**Table 2**). Like humans, there are four IgG subclasses, (IgG1, IgG2a, IgG2b, and IgG3) though the nomenclature differs between the species: for instance, IgG2a is most similar in its effector functions to human IgG1. The atypical Fc receptor TRIM21 is broadly expressed in the cytoplasm and possesses ubiquitin ligase activity. It can bind all classes of IgG (55) as well as IgA and IgM (56, 57). Following detection of intracellular immune complexes, TRIM21 stimulates a co-ordinated series of ubiquitination steps culminating in the degradation of immune complexes at the proteasome and an antiviral transcriptional response (56, 58–60).

The major site of Fc receptor expression in the brain is on the surface of microglia, the resident phagocytic immune effector cells of the CNS. In humans this includes the cell surface receptors FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa (62). There are reports of FcγRs on other cell types in the mouse brain, including on neurons (62, 63). However, other studies that sought evidence of FcγR expression at the transcript and protein level suggest that expression is minimal or absent in cells other than microglia (64, 65). The discrepancies between these findings may lie in the region of the brain analyzed as staining has been reported to be specific to regional neuron populations (66) or may reflect *ex vivo* vs. *in vivo* conditions. Outside the brain, FcγRI expression has been detected on sensory and motor neurons (67–69). TRIM21 is universally expressed and we have confirmed its expression in mouse primary neurons and human neuroblastoma cells (70). The neurodegenerating brain is an inflamed state, with widespread microglial activation and production of inflammatory cytokines including TNF, IL-6, and IL-1β (71, 72). Levels of TRIM21 and cell surface FcγRs are both increased following immune activation (58, 73). This is pertinent to the development of immunotherapeutics, as the degenerating brain may exhibit an

TABLE 1 | Human Fc receptors.

Name	Activity	High affinity ligands	Low affinity ligands	Peripheral expression	Brain expression
FcγRI	Activatory	IgG1, IgG3, IgG4		Mϕ, DC	MG
FcγRIIa	Activatory		All IgG subclasses	Mϕ, DC, Neutrophil, Basophil, MC, Eo, Pl	MG
FcγRIIb	Inhibitory		All IgG subclasses	B cells, Basophil, DC, Mϕ	MG
FcγRIIc	Activatory		All IgG subclasses	NK, Mϕ, Neutrophils	?
FcγRIIIa	Activatory	IgG3	IgG1, IgG2, IgG4	NK, Mϕ	MG
FcγRIIIb	Neutral		IgG1, IgG3	Neutrophils, Basophils	?
FcRn	Transcytosis, recycling	All IgG subclasses		Mϕ, DC, Neutrophil	BBB endothelium
TRIM21	Activatory/degradation	All IgG subclasses	IgA, IgM	Universal, high in Mϕ, DC, B cell	MG, neurons

Summary of the localization of expression and binding characteristics of human cell surface FcγRs, the recycling Fc receptor, FcRn, and the cytoplasmic Fc receptor TRIM21. High-affinity interactions are defined as those with an dissociation constant (K_d) $<10^{-7}$ M. MΦ, monocyte/macrophage; DC, dendritic cell; Eo, eosinophil; NK, natural killer cell; MG, microglia; BBB, blood-brain barrier. Information for this table adapted from Bruhns and Jönsson (50), Vidarsson et al. (51), and McEwan (61). ? denotes that CNS expression is not defined.

TABLE 2 | Mouse Fc receptors.

Name	Activity	High affinity ligands	Low affinity ligands	Peripheral expression	Brain expression
FcγRI	Activatory	IgG2a	IgG2b	Mϕ, DC	MG
FcγRII	Inhibitory		IgG1, IgG2a, IgG2b, IgE	B cell, Mϕ, Neutrophil, DC	MG
FcγRIII	Activatory		IgG1, IgG2a, IgG2b, IgE	NK, Mϕ, Neutrophil, DC	MG
FcγRIV	Activatory	IgG2a, IgG2b	IgE	Mϕ, Neutrophil	MG
FcRn	Transcytosis, recycling	All IgG subclasses		Placenta, Mϕ, Neutrophil, DC	BBB endothelium
Trim21	Activatory / degradation	All IgG subclasses	IgA?, IgM?	Universal, high in Mϕ, DC, B cell	Neurons, MG

*Summary of the murine Fc receptors, their binding partners and pattern of expression. As in **Table 1**, high-affinity interactions are defined as those with an dissociation constant (K_d) $<10^{-7}$ M. MΦ, monocyte/macrophage; DC, dendritic cell; Eo, eosinophil; NK, natural killer cell; MG, microglia; BBB, blood-brain barrier; ? denotes possible interaction not yet demonstrated.*

exaggerated response to immune complexes. FcR upregulation may enhance the effectiveness of any Fc-mediated clearance mechanism, but has the potential to drive inappropriate immune stimulation. Trials of passively-transferred antibodies against Aβ have reported ARIAs, which are caused by intracerebral oedemas or microhaemorrhages (74). This represents a clear safety issue for immunotherapies and limits the range of doses available to clinicians. These adverse events are potentially driven by microglial activation following engagement of antibody-bound Aβ assemblies by FcγRs. Of note, studies on tau, αS and Aβ have reported mechanisms of protection that do not rely on engagement of cell surface FcγRs (65, 70, 75–79). Current clinical trials are therefore testing monoclonal antibodies with modified effector functions as a means to preserve activity whilst diminishing adverse events (80, 81). The nature of these immunotherapies will be discussed further below, before a discussion on their likely mechanisms of action.

CLINICAL IMMUNOTHERAPY

Several immunotherapies that target proteins implicated in neurodegeneration have entered human clinical trials. They fall into two categories: those that attempt to induce protective immunity in the patient through vaccination (active immunotherapy) or the infusion of monoclonal antibodies (passive immunotherapy). Active immunotherapy has as its benefit the sustained production of antibody from few vaccine doses. However, there remain issues of variable or incomplete protection between individuals and a risk that side effects may be long-lasting or irreversible. Passive transfer of monoclonal antibodies permits precise control of dosing and the epitope targeted, avoids stimulating a potentially damaging T-cell response and can be withdrawn in the event of adverse effects. However, the large quantities of recombinantly produced antibody that need to be periodically infused in passive immunotherapy approches come with considerable cost implications.

Aβ Immunotherapies

The most advanced of the neurodegeneration immunotherapies are those that target Aβ (82), where 11 different approaches have reached clinical trials, seven of which are passively transferred monoclonal antibodies and four of which are active vaccination approaches (82) (**Table 3**). The first of these, AN1792, an active vaccine against full-length $Aβ_{42}$, was halted following the occurrence of meningoencephalitis in 6% of the study population, all of whom had mild to moderate AD (83). Post-mortem analysis of two patients who developed

TABLE 3 | Clinical immunotherapies in neurodegeneration.

Name	Immunotherapy type	Target	Company	Most advanced clinical trial ID	Phase of trial
IMMUNOTHERAPIES TARGETING αS and TAU					
AADvac1	Active	Tau 294–305	Axon Neuroscience	NCT02579252 (mild AD)	Phase II
ACI-35	Active	Tau pS396, pS404	AC Immune & Janssen	ISRCTN13033912 (mild to moderate AD)	Phase Ib
BIIB054	Passive	α-synuclein	Biogen, Neurimmune	NCT03318523 (PD)	Phase II
BIIB076	Passive, huIgG1	Tau	Biogen, Neurimmune	NCT03056729	Phase I
BIIB092	Passive, huIgG4	Tau N-terminus	Biogen & Bristol-Myers Squibb	NCT03068468 (PSP)	Phase II
				NCT03352557 (early AD)	Phase II
C2N-8E12	Passive, huIgG4	Tau 25-30	AbbVie & C2N Diagnostics	NCT02985879 (PSP)	Phase II
				NCT02880956 (early AD)	Phase II
PRX002	Passive, huIgG1	α-synuclein 118-126	Hoffmann La Roche, Prothena	NCT03100149 (early PD)	Phase II
RG7345	Passive	Tau pS422	Hoffmann La Roche	NCT02281786	Phase I (discontinued)
RO7105705	Passive, huIgG4	Tau	AC Immune SA, Genentech & Hoffmann La Roche	NCT03289143 (prodromal to mild AD)	Phase II
				NCT03828747 (moderate AD)	Phase II
LY3303560	Passive	Tau conformational epitope	Eli Lilly	NCT03518073 (early AD)	Phase II
JNJ-63733657	Passive	Tau mid-region	Janssen	NCT03375697	Phase I
UCB0107	Passive	Tau 235–246	UCB	NCT03464227	Phase I
SELECTED IMMUNOTHERAPIES TARGETING Aβ					
Solanezumab	Passive IgG1	Aβ (monomeric)	Eli Lilly	NCT02008357 (at risk of AD / mild AD)	Phase III
				NCT01760005 (fAD)	Phase III
Gantenerumab	Passive IgG1	Aβ (assembled)	Chugai Pharmaceutical, Hoffmann La Roche	NCT01760005 (fAD)	Phase III
				NCT03444870 (early AD)	Phase III
AN1792	Active	Aβ42	Pfizer, Janssen	NCT00021723	Phase II (terminated)
Aducanumab	Passive IgG1	Aβ (assembled)	Biogen, Neurimmune	NCT02484547 (early AD)	Phase III
Bapineuzumab	Passive IgG1	Aβ (assembled and soluble)	Pfizer, Janssen	NCT00998764	Phase III (terminated)

A summary of immunotherapies against tau and αS that have entered, or are soon to enter, clinical trials and selected immunotherapies against Aβ. PSP, progressive supranuclear palsy; AD, Alzheimer's disease; PD, Parkinson's disease; fAD, familial Alzheimer's disease.

meningoencephalitis indicated a T-cell mediated response was probably responsible for the inflammatory pathology. A second generation of active immunotherapies aims to target the N-terminus of the Aβ peptide, thereby avoiding a C-terminal T-cell epitope that may have been responsible for T-cell activation following vaccination with full-length Aβ (83). For the passive immunotherapies against Aβ, a reduction in Aβ PET biomarkers has been observed for gantenerumab, aducanumab, and bapineuzumab (46–48, 84). Safety issues mainly concern ARIAs, especially in carriers of the *APOE4* allele (85). ARIAs are likely due to the antibody decoration of Aβ plaques and the use of antibodies that preferentially bind Aβ monomers over fibrils (e.g., solanezumab) may therefore represent a mechanism to avoid them. Despite the evidence of target engagement, there is no evidence of clinical benefits for any of the drugs that have been tested in Phase III trails, which are powered to

test efficacy. As the Phase III trials conducted to date have been conducted in patient groups with established AD, their failure suggest earlier treatment may be critical for cognitive benefits. Future trials will test this hypothesis in populations with dominantly inherited dementias, or at risk of developing sporadic AD based on PET Aβ accumulation, using gantenerumab and solanezumab (86, 87). There therefore remains cause for hope in the targeting of Aβ in AD, but, if it is to be successful, it will likely require early intervention, a pre-requisite of which is predictive diagnostics.

Immunotherapy Against Cytoplasmic Proteins

Over the past decade, it has been repeatedly shown that active vaccination against tau or αS can alleviate the burden of pathology in the mouse brain (88–94). The mechanism of this immune protection is likely mediated by humoral immunity, as passive transfer of anti-tau antibodies is sufficient to confer a protective effect (95–100). This situation is reminiscent of viral infections, where the passive transfer of antibodies often confers sterile protection against infection (101, 102). Encouraged by the reductions in protein pathology, preservation of brain volume and ameliorations of behavioral metrics in mouse studies, clinical trials of tau and αS immunotherapies have commenced, or are planned (**Table 3**) (103, 104). We here summarize the therapies, and the rationale behind them, using the available pre-clinical and clinical data.

AADVac1

Following screening of antibodies that inhibited *in vitro* aggregation of recombinant tau, a monoclonal antibody, DC8E8, was identified with potent inhibitory activity (105). The epitope of this antibody is HXPGGG, a motif present in each of the four repeat domains of full-length tau. Passive transfer of DC8E8 was protective in a transgenic mouse expressing truncated human tau. This data was used to select an epitope for active vaccination (tau 294-305 KDNIKHVPGGGS), conjugated to keyhole limpet hemocyanin (KLH). In transgenic rats expressing truncated human tau, the vaccine was alum-adjuvanted and was found to confer a reduction in total and hyperphosphorylated tau species (106). Following these findings, human trials of the vaccine were commenced. A Phase I trial demonstrated that vaccination successfully induced an anti-tau immune response in 29/30 patients, which was biased toward an IgG1 response (107, 108). Phase II trials are underway in mild AD and primary progressive aphasia patients (109).

ACI-35

ACI-35 is a 16mer peptide comprising tau residues 393–408 with phosphorylation at S396 and S404 (91). This overlaps with the epitope of PHF1, an antibody widely used to detect pathological tau species (110). In the ACI-35 vaccine, the doubly-phosphorylated tau peptide is delivered in liposomes. Vaccination conferred a reduction in levels of soluble and insoluble tau phosphorylated at S396 in mice transgenic for human P301L tau (91). Protection against other phosphorylation sites of tau were not observed. Levels of insoluble tau were reduced but were not statistically significant by conventional criteria. The vaccine promoted the rescue of a clasping defect in the P301L tau transgenic mice but had no effect in the Rotarod test, a more demanding agility task. From a safety perspective, there was no observed influx of lymphocytes and no induction of astrogliosis. A Phase Ib clinical trial is currently taking place in AD patients with mild to moderate symptoms.

BIIB076

BIIB076 is a fully human IgG1, derived from Neurimmune's reverse translational approach, which mines antibody sequences isolated from humans. Little pre-clinical work has been published for BIIB076, though it was found to bind with subnanomolar affinity to human and cynomolgus macaque tau (111). When given to macaques it was found to reduce total and unbound CSF tau at the highest doses. A Phase I trial is under way.

BIIB092

Induced pluripotent stem cell-derived neurons prepared from familial AD patients secrete a series of truncated tau products that were termed eTau (112). eTau species consist of the N-terminal region of tau and run between 20 and 35 kDa by SDS-PAGE western blot. When added to primary cortical neurons, eTau caused neuronal hyperactivity and upregulated the expression of Aβ. The authors propose a model wherein secreted tau creates a destructive feed forward loop: Aβ drives tau pathology and secreted tau in turn upregulates Aβ. The antibody IPN002 binds the N-terminus of tau and neutralizes the effect of eTau. *In vivo*, it reduced levels of tau in CSF in P301L tau transgenic mice. IPN007, the humanized IgG4 version of the antibody, now renamed BIIB092, is being evaluated in Phase II trials in PSP and early AD patient populations.

C2N-8E12

C2N-8E12 is a humanized IgG4 version of the antibody HJ8.5 that binds with picomolar affinity to the N-terminal region of tau at residues 25–30 (95). In a seeding assay in human embryonic kidney cells (113), HJ8.5 exerted potent protection against tau seeds isolated from aged mice expressing human P301S tau (95). When chronically perfused into the ventricles, or delivered intraperitoneally to the same mouse model, HJ8.5 substantially reduced the extent of staining with antibodies specific for pathological tau and improved cognition (95, 96). Two Phase II clinical trials of C2N-8E12 are currently in progress in PSP and early AD cohorts.

RG7345

RG7345 is a humanized rabbit monoclonal that targets a C-terminal epitope of tau phosphorylated at S422 (100). The antibody was found to specifically enter neurons that contained hyperphosphorylated tau, suggestive of a target-dependent uptake mechanism. When injected to the periphery of TauPS2APP mice, which express human P301L tau and mutant forms of APP and PSEN2, the antibody reduced levels of tau phosphorylated at S422. The drug entered a Phase I clinical trial that was completed in 2015. However, no results have been

posted for the trial and Roche discontinued development for unknown reasons.

RO7105705

Genentech and AC Immune published work demonstrating that a mouse IgG2b antibody that targets tau phosphorylated at S409 reduced pathological tau staining in P301L tau transgenic mice (65). Mutation of the D265A and N297G (DANG) residues of the antibody, which prevents binding of cell surface FcγRs but not to murine complement (114), did not substantially reduce protection but prevented release of inflammatory cytokines by microglia (65). This work likely informed the selection of the IgG4 backbone for RO7105705, which has reduced capacity to engage microglia when compared to other subclasses (115). The precise epitope of RO7105705 has not been disclosed though it was reported to target the N-terminus (116). Two Phase II trials in prodromal to mild AD and moderate AD cohorts are in progress.

JNJ-63733657 and UCB0107

Based on the rationale that antibodies targeting the termini of tau may ineffectively bind proteolytically-digested tau assemblies, Janssen and UCB have selected antibodies that target the mid-domain of tau. The antibodies both reportedly block seeded aggregation of tau in cell-based seeding assays (117). These antibodies both recently entered Phase I clinical trials.

LY3303560

Eli Lilly have humanized a well-characterized conformation-specific antibody, MC-1 (118), which binds a discontinuous epitope of tau comprised of the N-terminal EFE motif (residues 7–9) and the core region (residues 313–322). From cryo-electron microscopy structures, the EFE motif is hypothesized to interact with the core structure in the AD paired-helical and straight filaments (119). Accordingly, LY3303560 displays a preference for binding aggregated over monomeric tau (120). Little preclinical data have been published and the antibody subclass has not been disclosed. A Phase II clinical trial is currently under way in sufferers of early symptomatic AD.

PRX002

PRX002 is being developed by Roche and Prothena as an αS-targeting immunotherapy. The mouse monoclonal, 9E4, from which PRX002 derives, belongs to the IgG1 subclass (121). 9E4 targets a C-terminal epitope of αS (residues 118–126), with a preference of monomeric over assembled versions of αS (122). When repetitively delivered intraperitoneally to a mouse model that over-expresses wildtype human αS, 9E4 protected against neuronal cell loss and improved behavioral parameters including Rotarod (121). Levels of a C-terminal fragment of αS, and higher-order assemblies of αS, were reduced by passive vaccination. 9E4 was found to accumulate in neurons and co-localize with αS and with lysosomal (cathepsin D) and autophagosomal (LC3) markers. Consistent with induction of αS degradation via autophagy as 9E4's mechanism of action, the intensity of LC3 staining was increased following 9E4 treatment, and clearance of αS by 9E4 was prevented by inhibition of autophagy in neuronal cultures. Phase I trials reported favorable safety and pharmacokinetics with evidence of target engagement in serum (123). A Phase II trial is currently in progress in early PD patients.

MECHANISMS OF ANTIBODY-MEDIATED PROTECTION

Numerous mechanisms have been posited for how antibodies may deplete aggregated proteins in the brain. As with protection against viral infection, particular mechanisms likely dominate the activities of individual monoclonal antibodies. The selection of individual monoclonal antibodies for passive immunotherapy, and modulating antibody effector function, is therefore a means by which specific effector functions may be selected. We will here delineate the mechanisms that are likely to operate against protein aggregates and, where possible, relate them to pre-clinical and therapeutic studies.

Peripheral Sink

The peripheral sink hypothesis posits that antibodies binding to targets in the periphery will shift equilibrium dynamics across the BBB, thereby reducing the concentration of cerebral antigens (124). This provides a mechanism that would enable the depletion of cerebral antigens by administering antibodies that promote clearance in the periphery. If biologically valid, it would enable the problem of antibody penetrance to the brain to be bypassed, as maintaining peripheral pools would be sufficient to promote CNS antigen depletion. The application of the peripheral sink hypothesis has been mostly applied to Aβ, which can be readily detected in both CSF and serum and therefore potentially susceptible to this process. Solanezumab and ponezumab both selectively target monomeric Aβ, and have been proposed to operate via the peripheral sink mechanism (125, 126). Consistent with a transfer to periphery, both antibodies, as well as a monomer-binding monoclonal antibody, m266 which was given to mice, increased the serum concentration of Aβ (125, 127, 128). However, this may be an effect of prolonging the half-life of Aβ in serum by complexing with antibody, as concentrations of free Aβ were not diminished. In a non-human primate model, the enzymatic degradation of Aβ in the periphery, although efficient, did not reduce CNS Aβ load (129). This experiment implies that degradation of CNS antigens in the periphery is not sufficient to substantially reduce CNS load. However, it must be noted that anti-Aβ IgG may itself alter the rate of efflux of antigen from the brain by promoting export of antibody:antigen complexes and the enzymatic degradation experiment would not capture this effect. Local production of antibodies against proteopathic agents within the brain, or administration of antibodies directly to the CNS, is associated with high levels of protection (95, 130–133). This demonstrates that mechanisms that rely on direct CNS exposure to antibodies can dominate protective effects. It is therefore likely that in order to target CNS antigens effectively, the problem of low antibody concentration in the brain must be confronted head-on by ensuring sufficient intracerebral target engagement for therapeutic efficacy.

Neutralization

It has been known since the 1930s that the incubation of virus particles with antibodies often results in a reduction in infectious titer, a phenomenon termed neutralization (134). Given the mechanistic similarities between viruses and cytoplasmically replicating proteopathies such as tau and αS, there is value in comparing the effects of antibody on both types of pathogen. Neutralization can only effectively be studied in cell-based models, outside a living organism, as the professional immune system confounds observations. We will here extend established definitions of virus neutralization (135) to cytoplasmic seeded protein misfolding as:

> *the reduction in seeding potency observed following the binding of antibodies to proteinaceous assemblies in cell-based seeding or propagation assays in the absence of complement or cells of the professional immune system.*

This definition therefore excludes the effects of microglial clearance and other effector mechanisms that are likely to operate *in vivo*. The cellular substrate used for examining the effect of antibodies on seeding ability is typically mouse primary neurons, human cell lines, or, more recently, human neurons derived from induced pluripotent stem cells (136, 137). It remains to be determined whether the choice of cellular model influences the extent, and mechanism, of observed neutralization.

Antibodies that exert potent neutralizing responses against viruses in cell based systems frequently exert strong *in vivo* protection (135, 138, 139). Indeed, a neutralizing antibody response is considered a surrogate marker of protective immunity in many circumstances. Until recently neutralization was thought to be synonymous with preventing entry of viruses, or, more specifically, their genomes, to the interior of the cell (135). A post-entry mechanism of neutralization that relies on engagement of the intracellular Fc receptor TRIM21 has recently been characterized, and is discussed further below. For entry-blocking antibodies, though the end result is identical (viruses fail to enter the cell), there are numerous mechanisms by which this may be achieved. For example, antibodies may effect a block to entry by preventing engagement of cell surface receptors, agglutinating virus particles or blocking escape from endosomes, each of which ultimately results in a block to virus entry.

In proteopathic seeding experiments, antibodies have been documented to reduce or slow the uptake of tau to cells. Examples of these are the anti-tau monoclonal antibody HJ9.3 (79) and a polyclonal preparation against the tau C-terminus, which slowed the uptake of tau to iPSC-derived neurons (136). Likewise, the anti-αS antibodies Syn211 and Syn303 reduced the uptake of αS fibrils to mouse hippocampal neurons (78). Together, these findings demonstrate that entry blocking neutralization can operate against protein assemblies (**Figure 3A**). However, entry-blocking is by no means a universal mechanism, since the antibody HJ8.5, which potently neutralizes seeding (94), fails to block tau uptake to neurons (79). The N-terminal monoclonal antibody 5A6 (140) and a C-terminal polyclonal, BR134 (141), similarly exert neutralization activity without substantially preventing uptake (70). For these latter two antibodies, neutralization activity relies on intracellular neutralization via TRIM21. Further, without a firm understanding of the mechanisms of seed entry to the cell, it is not clear exactly how antibodies elicit a block to cellular uptake. For αS fibrils, interactions with the putative entry receptor LAG3 facilitate binding and uptake to cells (142). Inhibition of this interaction with anti-LAG3 antibodies C9B7W and 410C9 reduced αS uptake. For both αS and tau, interactions with sulfated proteoglycans promote aggregate uptake (143–145) and inhibition of this interaction is the proposed mechanism for HJ9.3 (79).

By analogy with viruses, it is conceptually possible that antibodies block entry to the cytosol at a post-uptake stage, for instance by blocking endosomal escape, or by promoting endolysosomal degradation. There has been little study on the ability of antibodies to act at a post-uptake, pre-cytosolic entry stage. Implementation of the necessary methods is technically challenging, and, as with approaches for viral infection, particles that have escaped to the cytoplasm must be reliably differentiated from the endosomal population, which is likely to be overwhelmingly greater. Surrogate markers of tau and αS entry to the cytoplasm, such as Galectin 3-GFP, which binds carbohydrates on disrupted endosomes, have been developed (146, 147) and could be usefully applied to the field of antibody neutralization.

For certain non-enveloped viruses, neutralization can occur entirely independently of entry blocking. Antibodies against adenovirus do not prevent entry to the cell but remain associated with viral particles in the cytoplasm. Once in the cell, antibodies are bound by the cytoplasmic, high-affinity Fc receptor TRIM21, which mounts a rapid degradation response against the immune complex (**Figure 3A**). This substantially reduces viral infectivity and genetic deletion of TRIM21 renders certain antibodies non-neutralizing (56, 60). Mice that lack TRIM21 are highly susceptible to viral infection and, unlike their wild-type counterparts, cannot be fully protected by passive transfer of neutralizing antibodies (148). The distinguishing feature of viruses that are susceptible to TRIM21 is that their capsids are naked (i.e., without lipid bilayer) and lack fusogenic or membrane pore-forming mechanisms that permit the separation of genomic material and antibody-bound antigens during entry. Rather, these TRIM21-sensitive viruses, which include adenoviruses and minor group rhinovirus (149), enter the cell through lysis of the endosome, leaving the antibody-bound virus particle exposed (150, 151). The uptake of naked protein assemblies and entry to the cytosol though spontaneous or aggregate-induced lysis of vesicles (146, 147), is, similarly, a route that allows access of antibodies to the cytoplasm. Indeed, several studies have found that antibodies are taken up with exogenously added tau seed, and that antibodies do not prevent tau uptake to neurons (70, 79, 96, 136). Antibody-coated tau assemblies that escape to the cytoplasm become associated with TRIM21, and are prevented from inducing seeded aggregation by its activity (70). The extent to which intracellular neutralization

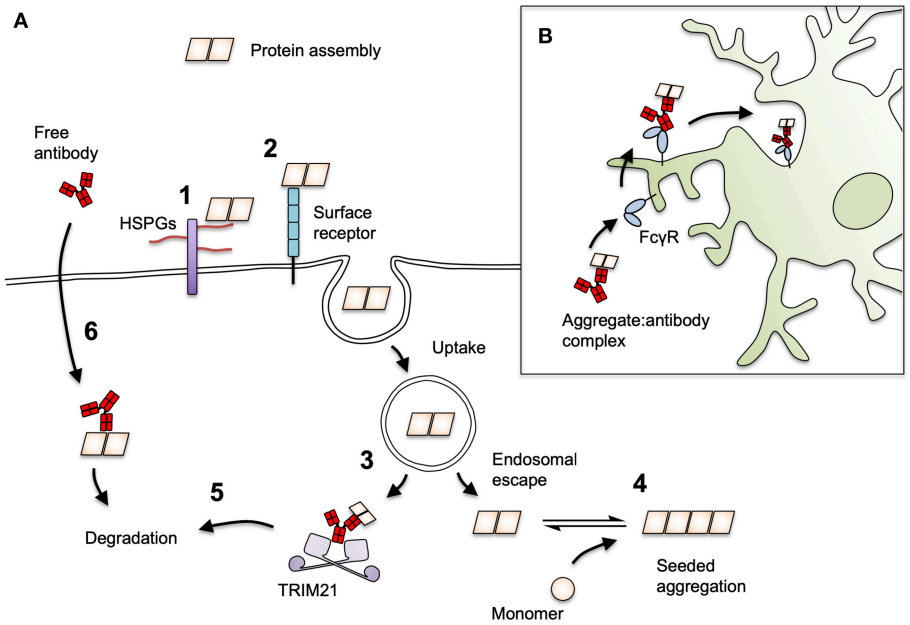

FIGURE 3 | Mechanisms of antibody-mediated protection against prion-like proteins. **(A)** The process of seeding for tau and αS may be neutralized by antibodies at several stages. Protein seeds attach to cells via interactions with (1) heparan sulfate proteoglycan (HSPGs) or (2) cell surface receptors such as LAG3 for αS. (3) Seeds must escape vesicular compartments in order to induce seeding, a step that by analogy with viral infection could be inhibited by antibodies. (4) Seeds that escape to the cytoplasm with antibodies attached may be prevented from undergoing seeded aggregation or (5) become targets for proteasomal destruction by the cytoplasmic Fc receptor and ubiquitin ligase, TRIM21. (6) Antibodies may be directly taken up into cells in a target-specific manner and mediate degradation of target proteins in the cytoplasm via TRIM21, or in the lysosome/autophagy pathways. **(B)** Antibody-decorated aggregates can be ligated by cell surface FcγRs on microglia. This induces their uptake and degradation and may play an important role in overall *in vivo* protection.

by TRIM21 contributes to the overall *in vivo* protection afforded by an antibody remains to be determined.

Clearance by Microglia

Microglia display an ability to take up naked assemblies of tau and αS and induce their degradation (152, 153). When in complex with antibodies, cellular uptake and degradation of both tau and αS is enhanced (79, 154, 155). This activity is Fc-dependent, as use of F(ab')2 fragments, which lack the Fc domain, or FcγR blocking antibodies, prevent clearance. This represents a mechanism that can be exploited for the therapeutic clearance of protein deposits (**Figure 3B**). However, FcγR-mediated clearance of protein deposits comes with a risk of activating a damaging immune response, as likely occurred during immunotherapy that targeted Aβ plaques (74, 85). Several immunotherapies have selected IgG4 as a scaffold with a rationale that it may minimize damaging pro-inflammatory responses (**Table 3**). However, as noted above, IgG4 binds FcγRs (50) and any reduction in inflammatory induction by IgG4 may owe more to its inability to fix complement (52, 53). Nonetheless a side-by-side comparison of an anti-Aβ antibody, MABT, with human IgG1 vs. IgG4 constant regions demonstrated a reduced ability of IgG4 to promote microglial inflammation by Aβ:antibody immune complexes (115). Two recent clinical trials with anti-Aβ IgG4 antibodies with reportedly low ability to engage FcγRs have commenced (80, 81). Uncertainty therefore persists in the selection of antibody isotypes for immunotherapy for maximal therapeutic effect and the extent to which isotype selection influences effector function in the brain. Passive immunotherapies on human IgG1 (BIIB076, PRX002) and IgG4 (BIIB092, C2N-8E12, RO7105705) scaffolds have been selected for clinical trials. Though an imperfect experiment, results of Phase II and III clinical trials, when considered together, will hopefully provide insight regarding the effect of isotype selection on therapeutic outcomes.

As noted above, an anti-pS409 tau antibody that possesses the DANG point mutations that prevent FcγR engagement retains the ability to prevent tau spread and neurotoxicity (65). Thus for antibodies that confer protection via alternative mechanisms, dispensing with FcγR engagement altogether provides a potential safety advantage. Other studies have reported that an antibody against pS404 of the mouse IgG2a isotype, which preferentially binds to activatory FcγRs (50), was more potent at clearing tau pathology than a mouse IgG1, which possess enhanced binding to the inhibitory FcγRII, despite targeting the same epitope with similar affinity (97). This would suggest that activatory microglial engagement, at least for these antibodies, has a net protective effect. Indeed, it has been argued that microglial engagement is both well-tolerated and therapeutically desirable (108). To satisfactorily address these issues, future work should determine the effect of antibody subclass on levels of *in vivo* protection by isotype switching monoclonal antibodies.

Intracellular Sequestration or Clearance

Free antibodies against tau have been found to enter neurons in cell based systems and in passive transfer experiments in mice (89, 99, 100, 156). Antibodies were found in complex with tau in the endolysosomal/macroautophagy pathways, suggesting that degradation is stimulated by antibody uptake. Antibody uptake could be blocked with antibodies against FcγRII/III in mouse neurons (99). The extent of this phenomenon is not clear, especially given the ambiguity concerning FcγR expression on the surface of neurons. The humanized antibody, MAb86/RG7345, was reported to enter neurons and was found associated with lipid rafts and intracellular or vesicular tau deposits (100). However, clinical trials for this antibody were discontinued for reasons that have not been disclosed. Intracellular sequestration is therefore a mechanism of action that is not explicitly represented in current clinical trials that target tau. It remains to be determined whether the phenomenon of intracellular antibodies involves the wholesale transfer of antibodies to the cytoplasm, or whether vesicles containing tau and antibody meet without cytoplasmic access. In the case of the former, it is expected that intracellular antibodies would be rapidly bound by TRIM21. It is therefore interesting that a monoclonal antibody, cis-113, specific to a soluble *cis*-tau conformer, was taken up by neurons and found to induce intracellular degradation of tau that was dependent on TRIM21 (157). Thus, both import of antibody in complex with tau seeds and uptake of free antibody by neurons may enable intracellular degradation of pathological protein species via the TRIM21 pathway (70, 157). Recent work demonstrates that TRIM21 can rapidly degrade diverse cellular proteins in experimental systems (158). It may therefore be possible to use antibodies and TRIM21 to specifically target disease-relevant protein conformations for degradation in the cytoplasm.

CONCLUDING REMARKS

The evidence that protein aggregation spreads in a prion-like manner is accumulating and compelling. The immune system is tasked with the detection and destruction of pathogens. In the case of tau, αS and other protein agents, these tasks are evidently not performed to a sufficient degree to resolve or limit aggregation. Notably, the detection of aggregated proteins as a threat is hindered by a lack of classical pathogen-associated molecular patterns, arising from their status as self proteins in an altered conformation. Destruction is hindered due to their physically robust and highly compacted nature, which is refractory to proteasomal degradation (159). Antibodies, either induced following active immunization or passively transferred, represent a means by which protein assemblies can be labeled as threats and then inactivated by neutralization, sequestration, or FcR-mediated effector functions. A deeper understanding of these mechanisms may provide a route to novel therapies in age-related neurodegenerative disease. Finally, the expense of long-term passive immunotherapy may ultimately prove prohibitive to its widespread clinical uptake. However, evidence of improvement in cognitive outcomes following immunotherapy would serve as a critical indicator that pathologically important processes have been targeted. In this way, passive immunotherapy may serve as proof-of-principle for future therapies, targeting the same processes, that are more suited to scaled production at affordable cost.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Neonatal Fc Receptor (FcRn): A Misnomer?

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Antibodies are essential components of an adaptive immune response. Immunoglobulin G (IgG) is the most common type of antibody found in circulation and extracellular fluids. Although IgG alone can directly protect the body from infection through the activities of its antigen binding region, the majority of IgG immune functions are mediated via proteins and receptors expressed by specialized cell subsets that bind to the fragment crystallizable (Fc) region of IgG. Fc gamma (γ) receptors (FcγR) belong to a broad family of proteins that presently include classical membrane-bound surface receptors as well as atypical intracellular receptors and cytoplasmic glycoproteins. Among the atypical FcγRs, the neonatal Fc receptor (FcRn) has increasingly gained notoriety given its intimate influence on IgG biology and its ability to also bind to albumin. FcRn functions as a recycling or transcytosis receptor that is responsible for maintaining IgG and albumin in the circulation, and bidirectionally transporting these two ligands across polarized cellular barriers. More recently, it has been appreciated that FcRn acts as an immune receptor by interacting with and facilitating antigen presentation of peptides derived from IgG immune complexes (IC). Here we review FcRn biology and focus on newer advances including how emerging FcRn-targeted therapies may affect the immune responses to IgG and IgG IC.

Keywords: IgG, IgG immune complex (IgG-IC), albumin (ALB), FcRn, immunity, therapeutic

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INTRODUCTION

It was F. W. Rogers Brambell who first proposed the idea of a fragment crystallizable (Fc) receptor system for Immunoglobulin G (IgG) after investigating the passage of maternal antibodies to fetuses and neonates (1). However, the identity of the specific receptor mediating this transfer, the neonatal Fc receptor (FcRn), remained unknown for nearly 30 more years (2) by which time other Fc gamma (γ) receptors (FcγR) had been identified (3–7).

As FcRn was structurally unique and not considered to be directly involved in immune responses, it was categorized as a non-classical FcγR that differs from the classical family members (**Box 1**) in several aspects (10). FcRn is distinctively a beta (β)-2-microglobulin (β_2m) associated protein that is structurally related to the major histocompatibility class I (MHC-I) family, yet it is unable to present antigenic peptides to T cells (11). Further, FcRn has a quasi-ubiquitous expression

BOX 1 | Classical FcγRs.

Protein family of Fc receptors for IgG (FcγRs) which are broadly expressed by cells of hematopoietic origin. Can be divided into inhibitory (FcγRIIB) and activating receptors (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB). Through binding of IgG via the Fc portion, FcγRs are essential for regulating responses to infections and controlling inflammation (8, 9).

pattern, possesses a predominantly intracellular localization, is monomorphic, and binds another, structurally and functionally unrelated protein to IgG, namely albumin (12). While the subtypes of IgG are fundamental in immune responses, albumin functions as a carrier protein in addition to being an important regulator of oncotic blood pressure (13). Despite these differences, IgG and albumin are the two most abundant serum proteins that possess a long serum half-life owing to their interaction with FcRn, which rescues them from intracellular degradation through a cellular recycling mechanism. Another of FcRn's functions is to transport IgG from mother to offspring thereby providing to the naïve and immature immune system of the newborn the experience and protection developed in the adult progenitor. This process is developmentally regulated in that it occurs antenally in rodents and humans through the inverted yolk sac or placenta, respectively, but uniquely continues at significant levels in the early post-natal life of rodents due to the high levels of FcRn expression in the intestinal epithelium. This functional expression of FcRn and its ability to transcytose IgG is not limited to the newborn but persists throughout life and permits the targeted delivery of IgG to sites where the presence of this type of antibody reinforces immunity, a process widely exploited by IgG-based therapeutics. Finally, the functions of FcRn are differentially determined by whether IgG is a single molecule, and thus monomeric, or present as an immune complex (IC). In the latter case, FcRn has been shown to critically regulate the innate immune responses as well as processing and presentation of antigens contained within IgG IC.

Here we review the versatile functions of FcRn in relation to albumin, monomeric IgG and IgG IC at different body sites. These observations have led to the emergence of protein-based therapeutics designed to harness, and in some cases, target FcRn function to promote the delivery of these therapies across mucosal barriers, increase their circulating half-life, or to treat IgG and IgG IC mediated diseases.

FcRn STRUCTURE AND BINDING OF LIGANDS

As an atypical FcγR, FcRn is structurally related to MHC-I molecules with a 40 kDa alpha (α) heavy chain that non-covalently associates with the 12 kDa light chain β_2m (14–16). The FcRn heavy chain consists of three extracellular domains (α1, α2, and α3), a transmembrane domain and a cytoplasmic tail of 44 amino acids (16). Since the first crystal structure of FcRn was solved by Burmeister and colleagues (14, 17), several other published crystal structures have shown that the α1 and α2 domains form a platform of eight antiparallel β-strands with two α-helices on top while β_2m is non-covalently associated with the α heavy chain (14, 18–22) (**Figure 1A**). Given its high similarity to MHC-I, FcRn was initially believed to present peptides (26), however the peptide binding groove of FcRn was subsequently found to be occluded (14). Instead this unusual FcγR binds with high affinity to IgG and albumin through non-overlapping sites at mildly acidic pH of 5.0–6.5 and exhibits no detectable binding to most of these ligands at neutral pH (**Figure 1B**), the exceptions being mouse IgG2b and some human IgG3 allotypes that display weak binding at neutral pH to mFcRn and hFcRn, respectively (27, 28).

FcRn interaction with the Fc portion of IgG occurs at the CH2 and CH3 domain interface, and involves the IgG Fc residue Ile253, and two central histidines: His310 and His435. Due to their pKa, the histidine residues become protonated at pH ∼6 which allows for interaction with the FcRn residues Glu115 and Asp130 (**Figures 1A,B**). As the pH increases above 6, histidine protonation is gradually lost which explains the pH dependence of the interaction (23, 29, 30). In addition to the heavy chain interactions, β_2m also forms contacts with IgG through the Ile1 residue (31). Mutating the IgG residues Ile253, His310, and His435 (IHH) leads to complete abrogation of FcRn binding at pH 6, which explains the reduced transcytosis and recycling of this mutated variant (32, 33). The FcRn binding site on IgG is distinct and distant from the binding site for classical FcγR which requires the glycosylation at the Asn297 residue of the Fc region of IgG (34).

Given that IgG is a homodimeric molecule, and contains two Fc domains, FcRn-IgG interactions have been proposed to occur with a stoichiometry of two FcRn molecules per one IgG (2:1). Indeed, an FcRn dimer was observed in crystals of the apo-FcRn (14), and in an FcRn-Fc complex (17, 35). Initially, the two binding sites on IgG for FcRn were not considered to be equivalent (35–37). Further studies with heterodimeric IgG, whereby only one of the sides of the Fc region was able to bind FcRn, showed reduced transepithelial transport in a model cell line (38). More recently it was shown that FcRn binds with equal affinity to each of the homodimeric wild-type (*WT*) IgG (39), but that the avidity effect resulting from the 2:1 complex formation was important for half-life extension (39). These results suggest that functional interaction of FcRn with monomeric IgG occurs with a 2:1 stoichiometry.

Recent work has indicated that in addition to the core Fc binding site on IgG, the fragment antigen binding (Fab) arms are also involved in FcRn binding (40–43). This was first suggested by experiments where antibodies with identical Fc but different Fab regions showed different affinity for FcRn and circulating half-life (40, 41). Accordingly, it was noted that the charge distribution of the Fab region, and the isoelectric point of the IgG itself can affect the dissociation from FcRn at physiological pH (41). As a result, a decrease in the rate of IgG dissociation from FcRn at physiological pH caused in faster *in vivo* clearance (41, 42, 44). Investigation of the FcRn IgG binding by the hydrogen deuterium exchange method has suggested a two-pronged interaction, involving direct interfaces between not only the IgG Fc region but also the Fab regions

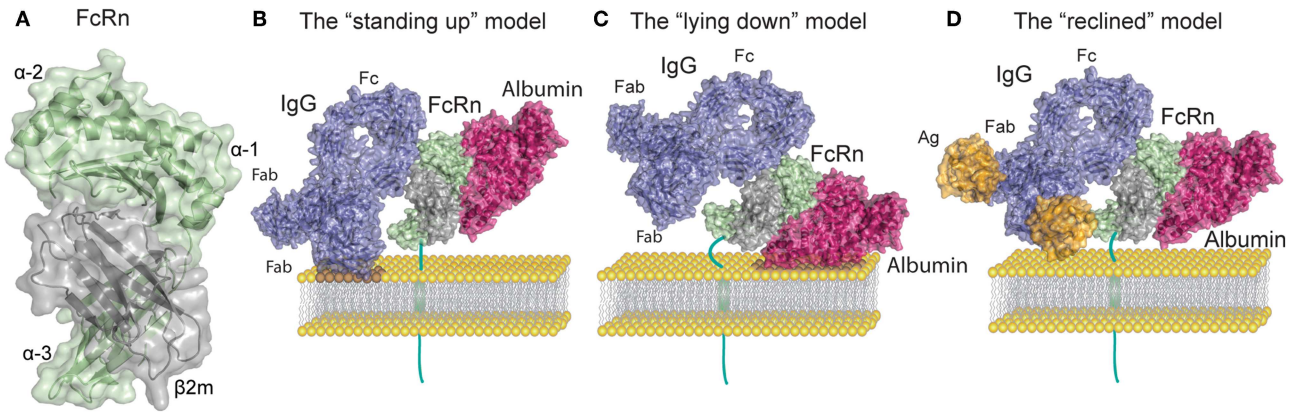

FIGURE 1 | FcRn structure and ligand binding. **(A)** Human FcRn heavy chain (green) is non-covalently associated with beta-2 microglobulin (β_2m, gray). **(B)** Topological representation of membrane associated FcRn in the standing up position. Bound albumin (magenta) and a monomeric IgG1 (blue) are modeled onto the structure of FcRn in complex with the Fc part of IgG^{MST} and albumin. In this orientation possible clashes may occur between the Fab arms of the IgG and the membrane. For simplicity the figures depict stoichiometric FcRn and IgG ratio of one to one. **(C)** Topological representation of membrane associated FcRn in the lying down position. This orientation accommodates Fab arms of IgG yet it potentially confines the albumin binding site. **(D)** Topological representation of membrane associated FcRn bound to albumin and IgG in complex with a small antigen [guinea fowl lysozyme (orange)]. The reclined orientation of FcRn on the surface of the endosomal membrane may most proficiently accommodate both ligands. Binding of large IgG IC (not depicted) might impose a lying down FcRn position. The figures were made using Adobe Illustrator, PyMol and the crystal structure data from: FcRn in complex with the Fc part of IgG^{MST} and albumin (23), a full-length human IgG1 antibody (24), and guinea fowl lysozyme (25), PDB IDs 4N0U, 1HZH and 1FBI respectively.

with FcRn (43). Nevertheless, surface plasmon resonance (SPR) studies with immobilized receptor could not detect differences in FcRn binding kinetics for IgG variants with different variable domains and different isoelectric points (39). Therefore, although it is incontestable that Fab regions can affect IgG binding to FcRn, the details of this involvement remain enigmatic.

Compared to IgG, binding of FcRn to albumin involves a larger surface area of the receptor, which is also more hydrophobic in nature than the IgG binding surface (23, 45, 46). Although this binding site for albumin on FcRn is located on the opposite side relative to that of IgG, it also relies on key histidine residues that bestow pH dependency to albumin-FcRn interactions (23, 47, 48) (**Figure 1B**). Albumin is a globular transport protein consisting of three structurally similar and highly flexible domains (49). Domain I (DI) and Domain III (DIII) are involved in its interaction with FcRn (**Figure 1B**). The main FcRn binding site consists of two hydrophobic pockets in albumin DIIIA and DIIIB that allow for binding of two FcRn tryptophan residues (Trp59 and Trp53) (23, 45, 50). Human albumin DI interacts with FcRn via two exposed loops that modulate FcRn binding (50, 51). However, similar participation of DI has not been observed for murine albumin, as this domain displays negligible contacts with mouse FcRn (52). Further, His166 of human FcRn (corresponding to His168 of mouse FcRn) is crucial for this interaction, and alanine substitution of this residue abolishes albumin binding (48). This occurs because at mildly acidic pH, His166 forms intramolecular hydrogen bonds that constrain the position of the loop containing Trp59 and Trp53, which are needed for albumin binding (23, 45, 48, 51). Several other histidine residues (His464, His510, His535) and Lys500 in albumin are also important for the interaction, and mutating any of these, reduces its binding to FcRn (47). Unavoidably, FcRn contact sites on albumin are also the binding sites for albumin cargo such as fatty acids, thyroxine, and drugs, as has been reviewed in (53). Thus, albumin molecules carrying long chain fatty acids exhibit reduced binding to FcRn (45, 54). These observations suggest that failure of albumin binding to FcRn may be used to optimize albumin-cargo delivery into cells due to decreased albumin recycling, in addition to its detrimental effects on the half-life of cargo-bound albumin. The diversity of albumin interactions with its cargo adds complexity to the mechanisms underlying albumin half-life and suggests a cellular mechanism for how albumin loaded compounds are delivered to cells.

In vitro protein-protein interactions and crystallographic studies of FcRn bound to albumin and IgG Fc have both shown that the receptor can engage its two ligands simultaneously, which is in line with the fact that the binding sites are non-overlapping (23, 55) (**Figure 1B**). Nonetheless, studies that only rely on soluble FcRn forms, without assessing surface immobilized receptor binding, as well as *in vitro* cellular assays or *in vivo* studies should be taken with caution. Early crystallographic data from the Bjorkman laboratory has put forward two putative models of FcRn Fc binding, wherein FcRn assumes either a perpendicular ("standing-up") or supine ("lying-down") position relative to the membrane (11, 17) (**Figures 1B,C**). Due to expected collisions between the Fab arms and the membrane surface inherent in the former model, it was considered less functional. It is interesting to note that mouse MHC-I molecules have been shown to exist on the surface of cell membranes in the lying down position, supporting the latter model (56). However, such an orientation of FcRn might render the albumin binding site of FcRn difficult to access. A recent study by Booth et al. highlighted the physiological relevance of

membrane-bound FcRn orientation, and illustrated that upon binding to monomeric IgG, FcRn may direct the antibody into a T-shaped conformation to allow for minimal steric hindrance with the membrane bilayer (57). Such a scenario is enabled by the marked flexibility of the Fab domains of IgG (58, 59), which can assume many different positions relative to the Fc (60–63), and also modulate FcRn binding (40, 41). Given these spacial restrictions an intermediate, "reclined" position of membrane-bound FcRn may be more likely to accommodate both IgG and albumin binding when compared to the "standing up" or the "lying down" models (**Figures 1B–D**). All these factors are particularly important when considering FcRn interactions with IgG IC, where Fab arms bound to antigen forming large IC may encounter even stronger steric effects.

When looking at the available binding affinities of FcRn to its ligands, diverse quantitative measurements have been published for these interactions (11, 28, 34, 35, 48–50). Consequently, to assess these reports one must consider the different experimental designs, natures of assayed reagents, as well as variability within the ligands themselves. SPR studies at acidic pH have reported that the K_D value for the human FcRn-albumin interaction (∼1 μM) is around 7-fold higher when the receptor is immobilized as compared to a design utilizing albumin immobilization (∼0.2 μM) (50, 55, 64). For the human IgG-FcRn interactions at acidic pH, the SPR-derived K_D is even more sensitive to the experimental setup, as the affinities reported when IgG1 is immobilized vary from ∼0.2–2.3 μM, whereas the values are in the nanomolar range when FcRn is immobilized (∼10–100 nM) (39, 65–68). The latter values are likely affected by the avidity effect from IgG's two binding sites for FcRn. One recent study compared albumin and IgG binding to FcRn in solution using microscale thermophoresis, which gave a K_D of 0.9 and 0.5 μM for albumin and IgG, respectively (69). Furthermore, diversity in binding affinities is also seen between FcRn and IgG or albumin from different species or across species (27, 68, 70). Thus, caution should be taken with the extrapolation of animal models to hFcRn and IgG or albumin interactions and vice versa.

CELLULAR TRANSPORT MECHANISMS

The pH-dependent ligand binding is crucial for all FcRn functions: including recycling and transcytosis, which allow FcRn to salvage its ligands from intracellular degradation pathways, to transport them across cell layers, and to potentiate efficient immune responses to antigen in the case of IgG IC.

The understanding of the central role of FcRn as a homeostatic regulator of circulating levels of IgG and albumin derives from studies in mice with conventional (*Fcgrt*$^{-/-}$) (71) or conditional (*Fcgrt*$^{fl/fl}$) (**Box 2**) (73) deletion of the FcRn heavy chain gene (**Figure 2**), although as a correlate, the β_2m light chain deficient mice (B2*m*$^{-/-}$) were also initially utilized (46, 74–78). Importantly, no human case of FcRn heavy chain deficiency has ever been reported, and the only clinical data available regarding the effects of FcRn deficiency in humans, comes from investigations of a rare human syndrome called familial hypercatabolic hypoproteinemia (79, 80). Affected individuals carry a mutation in β_2m that prevents cellular expression of β_2m protein and its associated heavy chains, including FcRn (81, 82). Normally the half-life of human IgG and albumin is around 19–21 days, while most other serum proteins, such as IgA, have half-life of ∼5–7 days at the longest (83). In the case of familial hypercatabolic hypoproteinemia two described patients had significant reductions in both IgG and albumin serum levels, with IgG and albumin half-lives of ∼3 and ∼6 days, respectively (80). In *WT* mice, the half-life of albumin and IgG was observed to be ∼39 and 95 h, respectively, compared to 25 h for IgA (46). The deletion of murine FcRn heavy chain, resulted in significant reduction of IgG and albumin half-life to ∼22 h (46), with concomitant decrease in circulating levels of IgG and albumin from ∼1.5 to 0.5 mg/ml and from ∼45 to 20 mg/ml, respectively (46, 66, 71, 75, 77, 78).

BOX 2 | Cre–lox recombination.

A site-specific recombinase technology that allows DNA modification targeted to a specific tissue or cell type, or to be triggered by a specific external stimulus. Relies on the DNA recombinase Cre and its recognition (loxP) sites. For conditional mutagenesis a target gene is modified by the insertion of two loxP sites that enables excision of the flanked (floxed) gene segment by Cre-mediated recombination. The floxed strain can further be crossed with a Cre transgenic line resulting in target gene inactivation *in vivo* within the expression domain of Cre (72).

The mechanism underlying these FcRn-mediated effects on IgG and albumin half-life is the pH-dependent diversion of both ligands from intracellular degradation pathways. This FcRn-dependent rescue of IgG from lysosomal degradation is a saturable process, such that administration of high doses of IgG (but not IgM, IgA or albumin) accelerates the clearance of endogenous IgG (84). Albumin injected into hypoalbuminemic individuals shows a half-life 4–5-fold longer than normal, which is in line with the rate of albumin salvage being also sensitive to FcRn saturation and expression levels (85–87).

The cell biological basis for intracellular recycling of IgG has been studied extensively by Ward et al., using mostly endothelial cell lines transfected with a fluorescently tagged FcRn (88–93). In these cells FcRn is known to localize intracellularly mainly to early endosomes positive for Rab5, EEA1 and recycling endosomes positive for Rab4 and Rab11a (**Box 3**) (90, 92). IgG is thought to enter endothelial cells non-specifically in pinocytocytic vesicles and subsequently bind to FcRn in EEA1-, Rab5-, Rab4-, and Rab11a-positive sorting endosomes characterized by pH of ∼6. IgG-bound FcRn then separates from sorting endosomes to Rab4- and Rab11a-positive recycling endosomes. The IgG variant His435Ala, which does not bind to FcRn, is instead sorted to lysosomes (89, 93). Recycling of FcRn bound IgG proceeds through multiple types of exocytic processes, including the fusion of Rab11a-positive vesicles that contain both FcRn and IgG with the plasma membrane for rapid release, or the so-called "prolonged release," where multiple pulses of IgG excretion can occur over a longer period of time (90). Intracellular trafficking-studies of IgG IC have shown that, whereas monomeric IgG and small IgG IC follow the

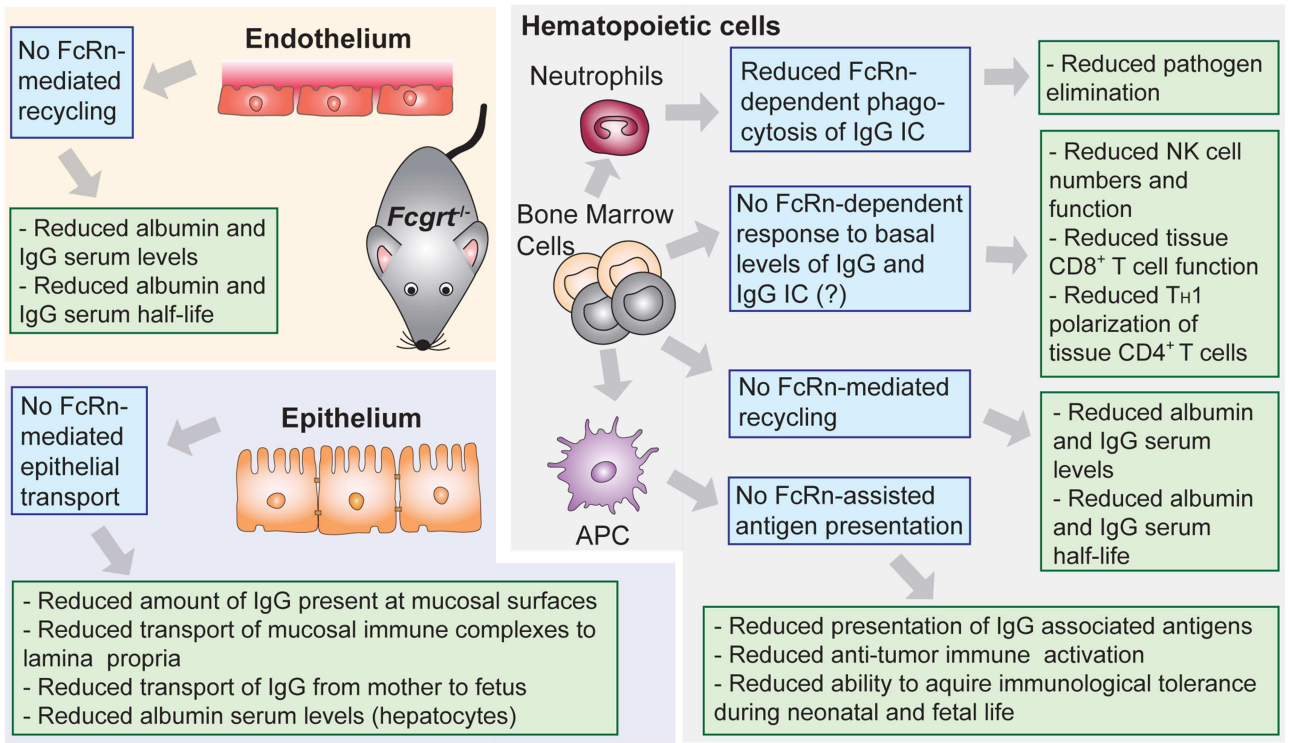

FIGURE 2 | Lessons from conventional and conditional FcRn deficient mouse models. Phenotypic observations reported in mice deficient for FcRn (Fcgrt$^{-/-}$). Blue boxes denote FcRn-affected function and green boxes denote pathophysiological consequences.

BOX 3 | Rab proteins.

Large protein family of small Ras-like GTPases that are regulators of vesicle trafficking in cells. They control vesicle budding, uncoating, fusion and membrane identity through recruitment of effector proteins (94, 95).

recycling pathway, large IgG IC are mainly sorted to lysosomal compartments (96). This has been shown in human monocyte derived dendritic cells (DC), in which FcRn transports IgG IC to degradative compartments (LAMP1^{+}) involved in antigen presentation (97). As the intracellular trafficking of FcRn has been mainly studied using IgG as a ligand, it is unknown whether albumin recycling is governed by the same principles. Further, intracellular sorting of albumin and IgG have not been directly compared in the same experimental system. Recent studies in a human endothelial cell line have however shown sorting of albumin to early endosomes positive for EEA1; the recycling of albumin was noted with variants having high FcRn affinity, and lysosmal sorting of albumin variants with low FcRn affinity (98, 99).

The FcRn transcytotic trafficking has mainly been studied using the model epithelial cell line from dogs, Madin-Darby Canine Kidney II cells (MDCK II). In this model, the cellular regulators of FcRn-IgG transcytosis differ from those involved in recycling, and FcRn mediated transcytosis in both directions requires both Myosin Vb and Rab25 (100). In addition, calmodulin, which can bind to the membrane proximal part of the cytoplasmic tail of FcRn in a calcium dependent fashion, is involved in this process (101). As is the case for recycling, endosomal acidification is also required for FcRn-mediated transcytosis (102–104). In MDCK II cells stably expressing human FcRn/β_2m, the receptor localizes mainly to apical vesicular structures and has been shown to traffic more frequently to the basolateral membrane, a process which relies on the presence of tryptophan and leucine residues in the cytoplasmic tail of FcRn (105–107). Electron tomography studies using rat intestinal epithelial cells have shown that clathrin is associated with the endocytotic and exocytotic processes involving FcRn, which supports the notion that it is rapidly retrieved from the plasma membrane after exocytosis (108). In contrast, rat FcRn displays opposite polarity when expressed in MDCK II, trafficking predominantly in a basolateral-to-apical direction. Such distribution depends on differences in receptor glycosylation, as rodent FcRn has four glycosylation sites and human has only one (109). Furthermore, using rat inner medullary collecting duct cells, transcytosis of rat FcRn in the apical to basolateral direction was shown to require phosphorylation of a serine residue (Ser313) in the cytoplasmic tail, whereas transcytosis in the basolateral to apical direction did not (110). Thus, the ligand-sorting and transcytotic functions of FcRn are mediated by specific regions and residues of the cytoplasmic tail of FcRn, which may differ between species.

Given that FcRn is a mostly intracellular receptor with functions that depend on its trafficking in the recycling and transcytotic pathways, surprisingly few studies have focused on

how FcRn's intracellular trafficking is regulated. This might vary according to cell type, nature of the specific ligand and its valence as well as its interplay with other receptors or regulators of intracellular trafficking.

FUNCTIONAL CONSEQUENCE OF FcRn EXPRESSION IN EPITHELIUM

As non-classical MHC-I family members are characterized by unique and more restricted expression patterns than classical MHC-I molecules, it was initially surmised that FcRn, mediating transport of IgG from mother to offspring, was only present in placental and intestinal tissues during the fetal and neonate period. Since then, however, FcRn expression has been detected almost ubiquitously in diverse tissues throughout the body including epithelia, endothelia and cells of hematopoietic (HC) origin. FcRn epithelial expression has been shown in the intestines (enterocytes) (102, 111, 112), placenta (syncytiotrophoblasts) (113), kidney (podocytes and renal proximal tubular cells) (114), and liver (hepatocytes) (115).

Intestinal FcRn

It was more than 40 years ago that Jones (116) and Rodewald (117) described age- and tissue-specific transfer of IgG in rodents. They illustrated that segments of the proximal jejunum but not ileum of 10–14-day old rats transported only IgG from the lumen to the circulation, which was non-detectable in 22-day old rats. Subsequently, the receptor responsible for this transport was isolated from the proximal small intestine of neonatal rats (2). Since then, studies in humans characterized FcRn expression at intestinal mucosal surfaces throughout life in both the small and large intestine, including villous and crypt enterocytes in addition to goblet cells and sub-populations of enteroendocrine cells (102, 111, 112, 118). In these cells, FcRn was located mainly intracellularly and on the apical membrane lining the gut lumen.

It is important to mention that in humans, little maternal IgG is transmitted to the neonatal circulation across the intestines, as most of humoral immune competency is assured by placental transfer. In contrast, FcRn-mediated uptake of IgG in rats and mice occurs both during the fetal and neonatal periods via transfer across the inverted yolk sac placenta and intestine, respectively. In cattle and pigs, the neonates rely entirely on postnatal uptake of colostral antibodies, mainly IgG, via intestinal epithelium for systemic humoral immune protection. These differences are also reflected in the levels of antibodies present in colostrum and milk, where IgG represents up to 3% of total antibody levels in humans as compared to 80% in cattle (119). Despite these species-specific differences, it is clear that FcRn consistently plays a central role in establishing humoral immunity in mammalian offspring.

While the evolutionary fitness afforded by FcRn in early life is apparent, its utility in adults to justify life-long expression in the intestine is less well-understood. Experiments in murine model systems have demonstrated that circulating monomeric IgG can be delivered into the intestinal lumen of FcRn humanized mice but not of *Fcgrt*$^{-/-}$ mice (120). Accordingly, IgG is present in mucosal secretions of the gastrointestinal, respiratory and genital tract where IgG antibodies together with IgA and IgM function together in host defense (121). However, while dimeric IgA and pentameric IgM is transcytosed unidirectionally via the polymeric immunoglobulin receptor, FcRn expressed in epithelial cells mediates transcytosis of IgG in both directions (105, 112, 120, 122, 123). Thus, FcRn in the intestines can deliver IgG into the lumen, and it also transcytoses monomeric IgG or IgG IC in reverse direction back into the lamina propria (**Figure 3**). This process ensures specific delivery of luminal antigens in the form of IgG IC to mucosal dendritic cells that can then regulate immune responses (120, 122). Indeed, the absence of FcRn results in greater susceptibility to mucosal infections with pathogens such as *Helicobacter pylori, Citrobacter rodentium,* or *Chlamydia muridarum* (122, 124, 125).

Although FcRn dependent transcytosis of IgG in the gut is well-established, the evidence for albumin transport has only recently been established. In initial studies, bovine serum albumin (BSA) conjugated to ferritin was not transported into the circulation of neonatal rats (117). On the other hand, Udall et al. showed that significant absorption of BSA occurred within first week of life in rabbits (126). More recently, investigations using MDCK II cells expressing human FcRn and β_2m showed bidirectional albumin transcytosis (54). Epithelial transcytosis of albumin was also reported in another *in vitro* study using Caco-2 cells (127). Given that very little albumin is lost in the gastrointestinal tract, it is possible that any proximal transport of albumin into the intestinal lumen might be compensated for by FcRn-reuptake or alternately by reabsorption. Such mechanisms might explain the progressive increase in FcRn expression levels from duodenum to proximal colon (112), as well as the presence of cubilin in human small intestine (128) (**Box 4**), which would allow for receptor-mediated uptake of albumin similar to processes occurring in the proximal tubules of the kidney (130).

As mentioned above, IgG IC are transported by hFcRn-expressing transgenic mouse gut epithelial cells in an inflammatory setting of *E. coli* infection (122). Notably, the original experiments of Rodewald utilized ferritin conjugated immunoglobulins, which are large protein complexes (117). Currently, studies in lactating female mice sensitized to different allergens during pregnancy have illustrated FcRn-mediated transport of IgG IC from breast milk across the gut epithelium (131–135). In this context, the transfer of antibody-antigen conjugates resulted in induction of tolerance to allergen in the offspring (131–133, 135). Recent reports have also supported the role of FcRn in intestinal transport of anti-IgE-IgG IC (136).

Placenta

In line with FcRn function in transferring IgGs from mother to neonates across the gut epithelium in rats, observations from other species, notably humans and rabbits, have shown prenatal transport of IgG across the placenta or yolk sac, respectively (137). The species-specific fetal or neonate transfer of IgG has mainly been explained by placental anatomy differences across species and the level of placental invasiveness (138). For instance, in ruminant epitheliochorial placenta, six tissue layers (maternal capillary endothelium, maternal uterine connective

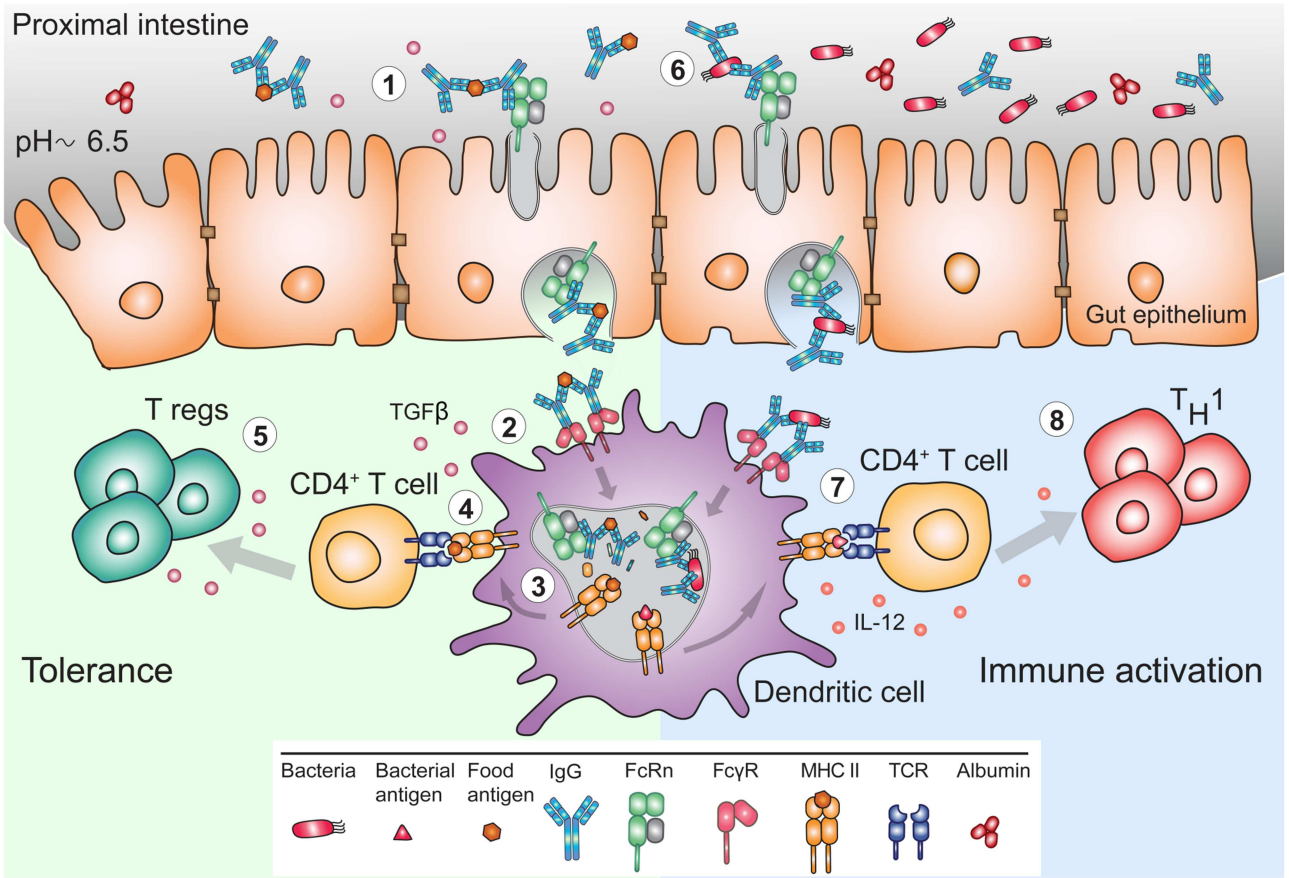

FIGURE 3 | FcRn mediates bidirectional transport and immune response to IgG and IgG immune complexes in the gut. (1) The pH of the mucosal surface of the proximal intestine can be slightly acidic, such that FcRn can bind maternal IgG and IgG IC already at the cell surface, and transcytose these to the basolateral side. (2) APC such as DC, can bind and actively internalize IgG IC via FcγR. (3) FcRn in APC assists in antigen processing and delivery of the IgG IC to antigen loading compartments where peptides derived from these complexes can be loaded onto MHC II for presentation to $CD4^+$ T cells. (4) In early life, presentation of antigen-derived peptide on MHC II in presence of other maternal milk-derived factors provides (5) tolerogenic environment to $CD4^+$ T cells. In these instances, FcRn expression by APC is crucial for induction of $CD4^+Foxp3^+$ regulatory T cells (Treg). (6) In adulthood, during infection, pathogen derived antigens bound by lumenal IgG will be transported across mucosal membrane in an FcRn-dependent manner and (7) delivered to APC, which process and present antigens, (8) for subsequent activation of immune responses.

tissue, uterine endometrium, trophoblast, embryonic connective tissue, and embryonic capillary endothelium) are interposed between the maternal and fetal circulations, while in the human hemochorial placenta three layers (trophoblast, embryonic connective tissue, and embryonic capillary endothelium) typically separate the two circulations. Thus, transport of IgG from mother to fetus in humans involves fewer cellular layers to traverse. Human FcRn has been found in both fetal endothelium and apically localized vesicles within the syncytiotrophoblasts that are in direct contact with maternal blood (113, 118, 139, 140).

IgG is the only antibody class that is transported across the placenta (141, 142), and this process is dependent on FcRn (66). Of the four IgG subclasses IgG1 and IgG4 are transported readily, whereas IgG2 and IgG3 show somewhat less efficient transplacental passage (28, 142, 143). In *ex vivo* human placenta transport studies, model IgG molecules disabled in FcRn binding did not cross to the fetal circulation (66, 144), while, conversely, an IgG variant with improved affinity for FcRn was transported more efficiently (145). Also, polarized human trophoblast-derived BeWo cells exhibited apical to basolateral IgG transcytosis and apical IgG recycling (146).

Classical FcγR (FcγRII and FcγRIII) have been detected in placenta and postulated to potentially participate in transplacental IgG transfer, whereas other studies could not find evidence for this (147–154). FcγRIIb2 is expressed in placental endothelial cells and FcγRIII in syncytiotrophoblasts (147–153). Pointing against involvement of FcγRs is the fact that an IgG3 variant with hinge-region deletions that prevents binding to all FcγR but retains FcRn binding was still transported to the fetus (154). Likewise, aglycosylated IgG variants that are unable to interact with FcγR, but bind FcRn, were transported in mice (155). Comparison of glycosylation patterns between fetal and maternal IgG showed that IgG transport was not glycosylation selective (143). In any case, the differential transport of IgG subclasses suggests that other factors in addition to FcRn may be involved in transplacental transport.

BOX 4 | Cubilin and megalin.

Cubilin is a large endocytic receptor responsible for intestinal absorption of the intrinsic factor vitamin B-12 complex, and renal tubule reabsorption of filtered plasma proteins including albumin, transferrin, vitamin D binding protein etc. Megalin (also known as LRP2) is another large endocytic receptor that belongs to a family of receptors with structural similarities to the low-density lipoprotein receptor (LDLR). Cubilin is a peripheral membrane protein that is dependent on megalin for efficient reabsorption in the kidney. Intestinal reabsorption of vitamin B-12 requires the protein amnionless, which is also needed for appropriate plasma membrane localization of cubilin (129).

In rodents, a major anatomical difference is the presence of chorioallantoic placenta as well as second inverted yolk sac placenta, where IgG transport is thought to occur throughout the gestation. This is supported by detection of FcRn in yolk sac endoderm and its absence in mouse chorioallantoic placenta (152). The crucial role of FcRn in transfer of IgG was demonstrated in offspring from heterozygous FcRn deficient mice. FcRn deficient fetuses displayed negligible levels of IgG compared to FcRn-heterozygous or *WT* littermates (152). Similar and efficient transplacental transfer of Fc-fusion proteins (such as Factor VIII-Fc) have been observed in mice (156, 157).

Transport of albumin across the placenta does not seem to occur to the same extent as for IgG. In a study from 1964, pregnant women in the last trimester of pregnancy were injected with radio-labeled IgG or albumin (158). While the levels of labeled IgG were found to be higher in the offspring than in mother's circulation, the levels of labeled albumin were only about 15% of the amount detected in the mother. It is still unknown why albumin and IgG are transported differently, but involvement of other albumin receptors could be part of the explanation. For example, megalin and cubilin (**Box 4**) have been found to be expressed in the placenta (159–161), and it has been suggested that they might facilitate retrograde recycling of albumin back to the maternal circulation (159).

Whether IgG IC cross the placenta in an FcRn dependent fashion is also less studied. By comparing concentrations of tetanus antigen and anti-tetanus IgG in maternal and fetal blood, Malek et al. observed that the ratio of antigen to antibody in the fetal circulation closely fit the maternal levels, suggesting transfer of IgG IC (162). In addition, May et al. illustrated placental transfer of IgG IC consisting of IgG and *Plasmodium falciparum* merozoite surface protein 1 (MSP1) from women in malaria endemic areas (163). More specifically, MSP1 was regularly detected in cord blood complexed to an antibody, and using an *ex vivo* human placental model, MSP1 IgG IC transport from maternal to fetal circulation was observed. MSP1 alone or with plasma from non-immunized individuals was not transported (163). Recent reports have also illustrated FcRn-mediated transplacental transport of maternal IgE through interactions with anti-IgE-IgG (164). First, IC in the form of anti-IgE-IgG bound to IgE were transported across polarized MDCK II cells in an FcRn dependent manner, and most of the IgE present in cord blood sera was found in complex with IgG (164). These studies indicate that FcRn mediates transplacental passage of not only monomeric IgG but IgG IC as well.

Elucidating the mechanisms behind the transport of FcRn ligands across the placenta will be crucial to understand immune responses occurring at the materno-fetal interface. In addition, it may provide knowledge to develop precision treatments targeting the mother or the fetus without reciprocally affecting the other. For example, in multiple fetal alloimmune diseases, including fetal thrombocytopenias and rhesus disease, preventing the transmission of maternal autoimmunity to the fetus may be transformative (165).

Kidney

Passage of proteins larger than 60–70 kDa into the urine is prevented by the charge- and size-selective filtration membrane in the glomeruli of the kidneys. Together with fenestrated endothelial cells, and the basement membrane, the kidney filtration barrier also consists of podocytes: large cells with foot processes that gate the basement membrane. Podocytes have been shown to express FcRn (114), and can transcytose IgG from the filtration membrane for delivery to the urinary filtrate (166). It is believed that this process serves two purposes: to clear IgG and IgG IC from the filtration membrane and to provide protective IgG to the urinary tract. Thus, *Fcgrt*$^{-/-}$ mice show accumulation of IgG in the glomerular basement membrane which subsequently can lead to serum-induced nephritis (166). FcRn in the kidney is important also for albumin homeostasis, as mice lacking FcRn have reduced serum levels of albumin, which can be rescued by transplantation of an FcRn expressing kidney (167).

The proximal tubule epithelial cells line the inside of the proximal tubules and are involved in reabsorption of proteins from the filtrate. FcRn in these cells has been shown to be involved in reabsorption of albumin and potentially IgG (130, 168), and one study demonstrated that the proximal tubule epithelial cells were involved in albumin reabsorption using inducible podocyte-specific tagged albumin expression (130). The reuptake of albumin from the glomerular filtrate also depends on the cubilin-megalin receptor complex (**Box 4**) which specifically endocytoses albumin from the renal filtrate, and delivers it to intracellular compartments where FcRn operates (130, 169–172). The functional interaction of FcRn with the cubilin-megalin receptor complex is an important mechanism of synergy between surface and intracellular receptors that are specific for albumin (130). Further work should investigate the interdependence between these receptors and address their potential interactions at different anatomical sites where both are expressed, such as the placenta and the intestine (99).

Liver

One of the important FcRn sites in the body is the liver (73, 118, 173). Indeed, the discovery of FcRn expression in adult rat hepatocytes was the first evidence that this receptor was expressed outside of the neonatal period (115). Since then, tissue expression of FcRn in humans, primates, rats, *WT* as well as humanized FcRn transgenic (TG) mice (174–176) has confirmed that the liver is a major site of FcRn expression, where its presence has

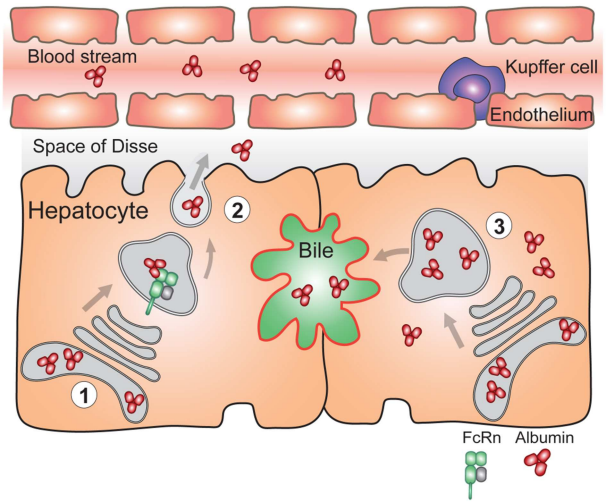

FIGURE 4 | FcRn in the liver is essential for vectorial delivery of albumin into the blood stream. (1) Hepatocytes are polarized epithelial cells of which the apical side (red) faces the bile duct, and the basolateral side (black) faces the fenestrated sinusoidal endothelium. The sinusoidal endothelium is populated by liver specific macrophages called Kupffer cells. Albumin is produced solely by hepatocytes. (2) FcRn in hepatocytes is required for delivery of newly synthesized albumin to the basolateral side of the cells, and subsequent secretion of albumin to the blood stream (left) (3) Absence of FcRn expression in hepatocytes results in increased albumin levels in the bile, its intracellular accumulation and lower circulating albumin levels (right). For simplicity, FcRn-mediated albumin recycling in hepatocytes is not depicted.

been detected in liver endothelium, liver sinusoidal epithelial cells (LSEC), Kupffer cells, hepatocytes and perhaps biliary epithelium (118, 173, 177). Using the human liver hepatocellular carcinoma cell line, HepG2, D'Hooghe et al. illustrated that the majority of FcRn is distributed intracellularly mostly in the early, late or recycling endosomes, and to a lesser extent in the trans Golgi network or lysosomes (178). The remaining small fraction of FcRn was present on the cell surface and could be subdivided into two pools: one that underwent rapid endocytosis and the other that was endocytosis resistant. The functional significance of this FcRn expression pattern is unknown. Furthermore, studies with human FcRn/β_2m^{TG} (*FCGRT*^{TG}) mice have also illustrated that FcRn was distributed intracellularly in addition to being associated with the sinusoidal and canalicular hepatocyte surfaces (54).

The relative contribution of hepatic FcRn to IgG or albumin biology is still emerging. On one hand, the liver eliminates complex macromolecules from the circulation such as IgG IC, while on the other, it produces albumin. Analyzing IgG biodistribution data from mice, rat, monkey, and humans, Shah and Betts showed that the liver contained ∼12% of the antibody attributed to the plasma compartment (179). Biliary excretion accounts for a very small amount of the eliminated IgG (54), and any hepatic IgG degradation that takes place likely occurs via intracellular catabolism in lysosomes. Studies in *WT* and FcRn deficient mice injected with antibodies labeled with either non-residualizing ^{125}I- or residualizing ^{111}I isotopes have illustrated a significant increase in IgG catabolism by the liver in the absence of FcRn (180, 181). Similarly, IgG antibodies that are unable to interact with FcRn are mainly catabolized in the liver, while *WT* antibodies are degraded mainly in the spleen, demonstrating that the liver possesses an important FcRn-mediated recycling capacity of monomeric IgG (182). Although hepatocytes were shown to efficiently recycle IgG Fc fusion proteins (183), absence of FcRn in hepatocytes did not significantly affect the circulating levels of IgG (54). Therefore, the specific cellular subset responsible for protection of monomeric IgG in the liver is still unknown. This is in contrast with small IgG IC that are eliminated efficiently from the circulation by the cells of the classical reticuloendothelial system (184, 185), mostly by the LSEC and also to some extent by Kupffer cells (186). This process relies on the expression of FcγRIIb, while the role of FcRn in LSEC has not been assessed (187).

As it pertains to the albumin homeostasis, both FcRn deficient humans (80, 81) and mice (46) are hypoalbuminemic. In *Fcgrt*$^{-/-}$ mice, the hepatic albumin production rate is paradoxically increased by ∼20% compared to normal mice, which is thought to represent a compensatory mechanism for the low circulating albumin levels (188). However, conditional deletion (**Box 2**) of FcRn in the liver which mainly affects hepatocytes (*Alb*^{cre}*FcRn*$^{fl/fl}$) resulted in inability to efficiently deliver albumin into the circulation. Thus, in the absence of FcRn, hepatocytes accumulated albumin intracellularly and biliary excretion of albumin significantly increased (54) (**Figure 4**). Using a polarized model cell line that co-expresses FcRn and albumin, it was shown that enhanced secretion of newly synthesized albumin occurred into the basolateral space modeling the bloodstream, rather than into the apical space which modeled the biliary ducts. Lack of FcRn resulted in mostly apical albumin secretion as well as intracellular accumulation (54). Thus, the presence of FcRn within hepatocytes mediates physiological albumin biodistribution through secretion of albumin into the circulation.

Altogether, FcRn expression in the liver serves two main purposes: to maintain monomeric IgG and albumin in the circulation and to direct albumin toward the circulation instead of to the bile. Whether removal of small IgG IC from the circulation also relies on FcRn expression by LSEC is unknown.

FUNCTIONAL CONSEQUENCE OF FcRn EXPRESSION IN ENDOTHELIUM

Endothelial cells line the entire vascular system and control the passage of numerous cells and molecules in and out of the circulation, and are one of the major cellular locations where FcRn controls the levels and persistence of IgG and albumin (83). Indeed, FcRn expression by these cells is well-documented in intracellular vesicular compartments (76, 118, 173, 189). As FcRn interactions with its ligands are restricted to intracellular acidic compartments, it is important to note that IgG is thought to be taken up by endothelial cells mainly by pinocytosis (88, 89, 92, 93), while albumin uptake is thought to be facilitated via binding to another albumin receptor, albondin (190). However, to our knowledge, this receptor has neither been sequenced, nor have

its functions been recently investigated. While a current study showed localization of internalized albumin in early endosomes and not to lysosomes, which is in line with FcRn mediated rescue from degradation (99), the precise albumin sorting mechanism has not been studied to the same extent as IgG.

It is important to remember that although albumin and IgG are the most abundant proteins in the circulation, two-thirds of total albumin and one-half of IgG reside in the extravascular compartment (191). Whether or not FcRn is involved in the above-mentioned distribution of IgG or albumin, and if so to what extent, is still unknown.

In vivo analysis of how FcRn contributes to IgG and albumin biodistribution via endothelial expression is currently lacking, although conditional deletion of murine FcRn in both the vasculature and cells of bone marrow origin (*Tie2*^{cre}) results in decreased IgG and albumin levels in the serum (73). The precise vascular location of FcRn remains to be determined, which is complicated by endothelial cell heterogeneity with differences between arteries, veins, large and small vessels, as well as diversity in microvasculature beds from different organs (192). Intracellular trafficking, recycling and transcytosis of IgG and albumin in the endothelia have so far mainly been carried out using cell lines, including human placental endothelial cells (HPEC), human umbilical vein endothelial cells, human dermal microvascular endothelial cells, or mouse SV40-transformed endothelial cells. Using polarized HPEC, it was shown that greater IgG recycling occurred at basolateral cell surfaces, representing the extracellular matrix, compared to the apical cell surfaces, which represents the blood vessel lumen (189). IgG transcytosis was consistently more prevalent in a basolateral to apical direction in HPEC. These results reflect the placental origin of the endothelia used in this study, in which IgGs are transported from maternal to fetal circulation across the endothelial monolayer. More recently, using non-polarized FcRn-transfected human umbilical vein- or dermal microvascular -endothelial cells, the recycling of both IgG and albumin was studied (69, 98, 99). The role of endothelial FcRn in handling IgG IC is not currently known, although as mentioned above LSEC are crucial in elimination of small IC from the circulation.

The Blood-Brain Barrier

The blood-brain barrier (BBB) restricts access of large molecules to the central nervous system (CNS) by separating the circulation from the CNS. Microscopy studies have shown that FcRn is expressed in brain microvascular endothelium as well as choroid plexus epithelium (193), where it has been suggested to mediate active transport of IgG from the brain into bloodstream (194, 195). In mice, intraperitoneal or intravenous IgG administration resulted in <0.01% of the injected dose to be detected in the brain (196), and at steady state, endocytosed IgG was localized to lysosomes within brain endothelial cells (197). Similarly, albumin is excluded from the CNS (198). In a mouse model of Alzheimer's disease it was shown that FcRn at the BBB was involved in removal of amyloid β-peptide-specific IgG IC (199). In rats, an IgG with improved FcRn affinity was cleared faster from the brain upon intracranial injection, than an IgG with no affinity for FcRn (195). Interestingly upon intra-cerebral injection the efflux of albumin from CNS is slow with an elimination half-life of 10–12 h, whereas IgG efflux is rapid with an elimination half-life of 48 min (194). Still, others have described a more limited role for FcRn in clearance of IgG from the brain (181, 200). Further studies are necessary to fully understand the role of FcRn in the BBB function, and attention should also be given to the choroid plexus epithelium which also expresses FcRn (193).

FUNCTIONAL IMPORTANCE OF FcRn EXPRESSION IN CELLS OF HEMATOPOIETIC ORIGIN (HC)

The description of FcRn expression after the neonatal period in adult liver was followed by a demonstration that FcRn is also abundant in cells of bone marrow (BM) origin in adult animals (201). Since then, FcRn presence in humans as well as several animal models including mice, rats and non-human primates has been shown. Thus, FcRn is expressed by monocytes, macrophages (both tissue resident and splenic), neutrophils, DC and B lymphocytes but not by T or natural killer (NK) cells (97, 118, 177, 201–205). However, due to the heterogeneity of these cell subsets, more detailed systemic studies are needed to precisely assess FcRn expression patterns and define species-specific differences. Regardless, the presence of FcRn mainly in antigen presenting cells (APC) indicates that it might provide functional benefits to these cells and directly implicates FcRn in IgG-mediated immune responses.

Overall, immunophenotypic analysis of *Fcgrt*$^{-/-}$ mice revealed subtle decreases in mucosal CD8^{+} T and NK cell frequency (205–207) as well as splenic CD8^{+} T cell frequency when compared to *WT* mice (205) (**Figures 2**, **5**). These cells also displayed functional defects; for instance, CD8^{+} T cells from the large intestinal lamina propria of *Fcgrt*$^{-/-}$ mice secreted less IFN-γ, IL-10, and TNF upon re-stimulation in comparison to *WT* littermates and exhibited inferior cytotoxic activity (206). In the absence of FcRn, NK cell development, maturation and function were impaired as well (205). Furthermore, lung-resident CD103^{+} DC, splenic macrophage and neutrophil subsets were increased (205, 207). As FcRn is not expressed by NK and CD8^{+} T cells, the observed defects were possibly associated with abnormal cytokine response of myeloid cells which affected the function of several other cell subsets in trans. Indeed, a similar defect in CD8^{+} T cells was present when FcRn was conditionally deleted in CD11c^{+} cells (*Itgax*^{cre}*Fcgrt*$^{fl/fl}$) (206). In addition, *Fcgrt*$^{-/-}$ DC exhibited decreased expression of IFN-γ, IL-12p35, T-bet, and TNF, which are all necessary for effective cytotoxic T cell-mediated immunity (206).

Further, it is noteworthy to contemplate the possibility that FcRn, a non-classical MHC-I molecule, may interact with one of the NK cell receptors that are acknowledged to bind classical and non-classical MHC-I family members (208). More importantly, in view of these results, studies of decidual NK cells, that are critical for the uterine spiral artery remodeling (209), become essential, given the documented importance of FcRn during fetal development.

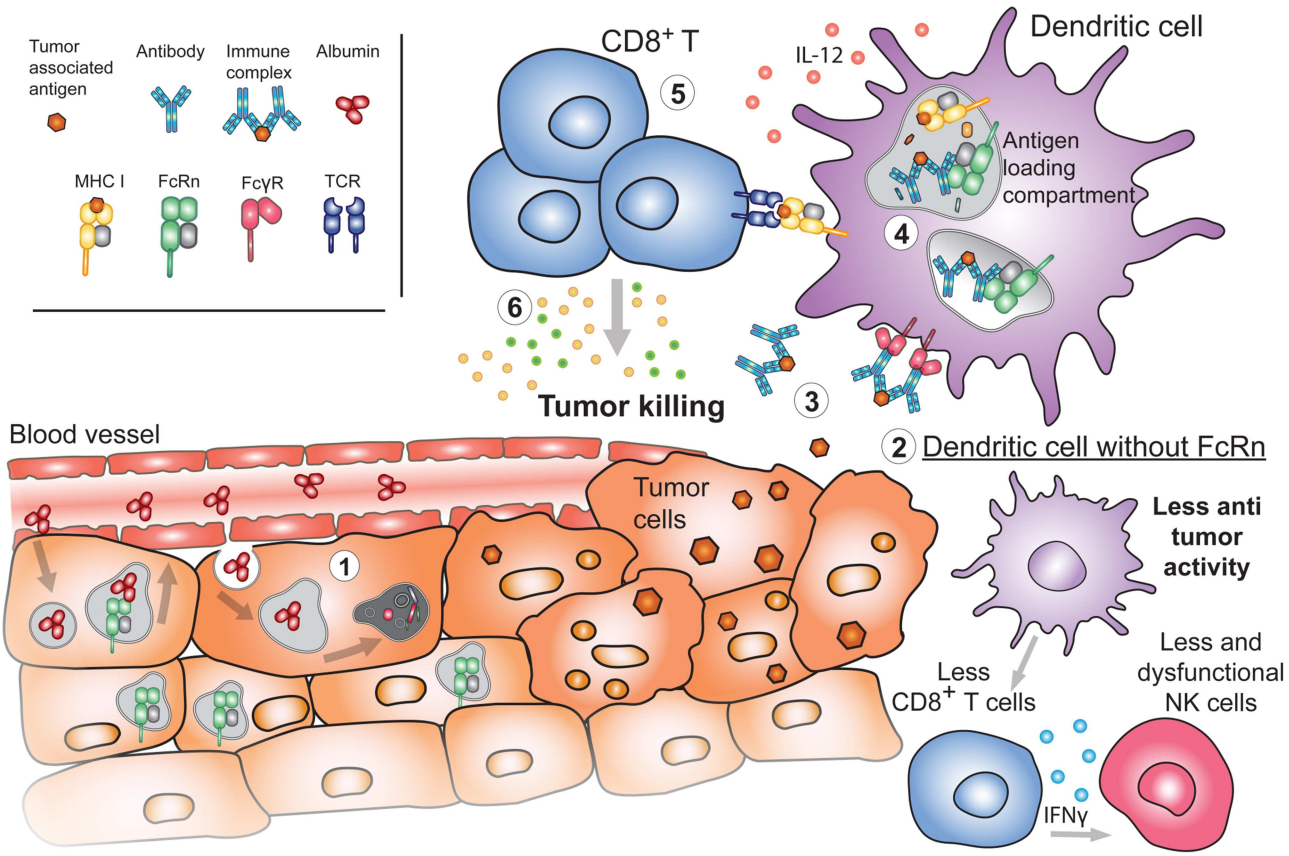

FIGURE 5 | Emerging roles of FcRn in cancer. (1) During the process of oncogenesis, cells can lose or downregulate FcRn expression. In these instances, tumor cells will be unable to recycle albumin upon its internalization. Albumin will instead be degraded, providing nutrients to the tumor and promoting tumor growth. (2) Absence of FcRn in APC may decrease the basal cytotoxic tone (such as IL-12 production) resulting in diminished numbers and function of either NK or $CD8^+$ T cells in tissues, generating a tumor-prone environment. (3) Released tumor antigens can be bound by antibodies and internalized by APC through FcγRs. Presence of FcRn in these cells is important for (4) the subsequent sorting and efficient processing of IgG IC in antigen loading compartments where tumor-derived peptides are loaded onto MHC I for cross-presentation to (5) cytotoxic $CD8^+$ T cells. Activated tumor specific $CD8^+$ T cells will (6) effectively target cancerous cells for destruction.

Outside of the intrinsic cellular consequences, up to now the major attention of the scientific community on the function of FcRn in *sensu stricto* immunity has focused on how it affects the half-life and biodistribution of IgG. Thus, the important contribution of the hematopoietic FcRn compartment to immunity *per se* was unanticipated. Nonetheless, this was demonstrated in a series of studies with BM chimeric mice whereby FcRn deficient, *WT* or $FCGRT^{TG}$ mice were used as donors and recipients interchangeably ($Fcgrt^{-/-}$ BM Donors → *WT* or $FCGRT^{TG}$ Recipients; *WT* or $FCGRT^{TG}$ BM Donors → $Fcgrt^{-/-}$ Recipients). Results of these studies showed that BM derived cells, in addition to vascular endothelial, epithelial, stromal and parenchymal cells, are necessary to extend the half-life of monomeric IgG in the circulation (97, 177, 210). Data from conditional deleted mice, where FcRn was absent either in vascular and hematopoietic ($Tie2^{cre}Fcgrt^{fl/fl}$) or CD11c ($Itgax^{cre}Fcgrt^{fl/fl}$) compartment corroborated these findings (54, 73). More recently, Challa and colleagues have described the effects of conditional FcRn deletion in macrophages or B cells and DC (211). They showed that the absence of FcRn in macrophages ($LysM^{cre}Fcgrt^{fl/fl}$), but not the latter cells ($CD19^{cre}Fcgrt^{fl/fl}$), results in excessive IgG degradation as IgG half-life and circulating levels were drastically reduced as compared to *WT* animals. Interestingly, in some instances the Cre recombinase activity is not exclusively operating in DCs ($Itgax^{cre}$) or macrophages ($LysM^{cre}$), and varies from tissue to tissue which can affect the level of deletion and perhaps affect other HC cells (212). Nonetheless, it is clear that FcRn deletion in cells of bone marrow origin decreases the levels and half-life of circulating IgG (54).

Similar types of experiments illustrated the contribution of HC to albumin homeostasis. Thus, on the one hand $Fcgrt^{-/-}$ BM chimeras ($Fcgrt^{-/-}$ BM Donors → *WT* Recipients) displayed lower circulating albumin levels when compared to *WT* mice (210). Still these levels were significantly higher than observed in complete $Fcgrt^{-/-}$ animals. On the other hand, reconstitution of $Fcgrt^{-/-}$ mice with *WT* BM (*WT* BM Donors → $Fcgrt^{-/-}$ Recipients) only partially restored circulating albumin levels (210). Furthermore, while conditional deletion of FcRn in vascular and hematopoietic ($Tie2^{cre}$) as well as

macrophage (*LysM*^{cre}) compartments resulted in about 2-fold lower albumin levels in the serum (73, 211), no changes were observed when FcRn was deleted in the CD11c compartment (*Itgax*^{cre}) (54). Thus, the deletion of FcRn in DC or other cells of HC origin does not affect circulating albumin levels to the same degree as IgG, and suggests that a considerable fraction of albumin recycling occurs primarily in non-HC compartments, although compensatory effects may also be at play.

Besides being a major site of monomeric IgG and albumin protection from degradation, HC expressing FcRn play an important role in immune responses to IgG bound antigens in form of IC. Indeed, experiments with FcRn$^{-/-}$ BM chimeras (*Fcgrt*$^{-/-}$ BM Donors → *Fcgrt*$^{+/+}$ Recipients) injected with small IgG IC have shown that the absence of FcRn in HC dramatically reduced the persistence of these complexes in the circulation (97). Larger IC formed by monoclonal anti-NIP IgG antibody and NIP-conjugated antigens (consisting of 15 NIP molecules per antigen) were cleared faster than small IgG IC but still were protected in an FcRn-dependent manner in HC (97). This is consistent with the observation in epithelial cell lines, that monomeric or small IgG IC are recycled while large IgG IC are diverted to late endosomes and/or lysosomes where they are retained for extended periods of time (96, 97, 213). In addition, FcRn cross-linking by IgG IC induces a signaling cascade that is associated with secretion of IL-12 and is overall skewed toward T helper 1 and T cytotoxic responses (206, 214). Given that low levels of circulating IC have been detected even in healthy individuals and animals (215–217), a basal amount of these IC might provide, via interaction with FcRn, a basal cytokine tone essential for HC homeostasis and explain the immunophenotype of *Fcgrt*$^{-/-}$ mice described above that include decreased inflammatory tone of HC as well as diminished NK and CD8$^+$ T cells functions (206). More importantly, in response to variable amounts of IgG IC, FcRn in HC would affect the outcome of an immune response.

Such responses are buttressed by the HC expression of low-affinity classical FcγR (FcγRIIa/b/c, FcγRIIIa/b) (**Box 1**) (10), which are mostly present at the cell surface, and interact with IgG IC rather than monomeric IgG, at neutral pH (218). Therefore, instead of pinocytosis or unspecific fluid phase endocytosis, HC are able to efficiently internalize IgG IC via receptor-mediated endocytosis which triggers particular signaling pathways (219), and may affect subsequent intracellular FcRn encounters with these FcγR-IgG IC complexes. It is well-recognized that FcγR triggered immune responses to IgG IC potentiate the processing of antigen contained within IC (220, 221). These can culminate either in MHC-I cross-presentation or MHC-II presentation to CD8$^+$ and CD4$^+$ T cells, respectively; however the degree and interdependence of FcRn in these processes are still emerging. Thus, mouse APC or human monocyte derived DC exposed to IgG^{WT} IC, but not to the FcRn non-binding variant IgG^{IHH} IC, induce greater CD4$^+$ T cell proliferation (97). CD8$^+$ T cell responses to cross-presented antigen contained within IgG IC are similarly dependent on FcRn, with one main difference. While the DC population that mediates cross-presentation of soluble antigens (CD8$^+$CD11b$^-$ DC) in mice exhibits little dependence on FcRn, the CD8$^-$CD11b$^+$ DC population relies significantly on FcRn to efficiently cross-present antigen contained within IgG IC and stimulate CD8$^+$ T cells both *in vitro* and *vivo* (214). In this instance, FcRn was important for movement of IgG IC into phagosomal compartments conducive to cross-presentation in addition to preventing their fast and excessive degradation in association with intracellular retention (214). In neutrophils, FcRn enhanced phagocytosis of IgG-opsonized bacteria and their delivery into phagolysosomes as compared to *Fcgrt*$^{-/-}$ cells (202). Interestingly, neutrophils treated with IgG IC that retained normal binding to FcγR but were unable to bind FcRn displayed reduced phagocytosis (202), suggesting that in some HC subsets classical FcγR and FcRn might function in parallel and not sequentially. The mechanisms underlying these observations need to be further established.

Dating back to Paul Elrlich (222) and F.W. Rogers Brambell (137), the initial impetus to study and understand passive immunity was the protection of the offspring from infection. Nevertheless, accumulating evidence illustrates that FcRn participates not only in the transfer of protective immunity but tolerance as well (223). Studies of murine materno-fetal and neonatal IgG transport clearly illustrate that FcRn plays an important role in induction of tolerance, however whether this effect is dependent on FcRn within HC was unknown until now (131). Using Fc-fused hemagglutinin and T cell receptor TG mice specific for hemagglutinin, Gupta et al. have illustrated that FcRn-dependent transplacental transport of Fc-hemagglutinin induced tolerance via antigen-specific regulatory T (T_{reg}) cells (156). In a similar type of experiment, the administration of Fc fused preproinsulin to pregnant mice resulted in efficient passage of these chimeric proteins to fetuses and prevented development of autoimmune diabetes. More specifically, Fc-preproinsulin was carried to the thymus by migratory DCs and provided support for the emergence of antigen-specific thymic-derived CD4$^+$ T_{reg} cells as well as induced development of impaired cytotoxic CD8$^+$ T cells (157). Furthermore, in an allergic airway disease model, it was shown that post-partum exposure of lactating female mice to airborne antigens led to decreased airway hyper-reactivity only in breastfed offspring, which was associated with the presence of TGF-β as well as IgG IC in the milk (133). Thus, it was the FcRn-mediated IgG IC transfer to the newborn that induced antigen-specific Foxp3$^+$ T_{reg} cells (132). Still, these studies mostly emphasized FcRn as a transplacental delivery receptor of IgG IC that permitted antigen delivery to APC, without specifically investigating FcRn's role within these cells as a mediator of tolerance. More recently, in an epicutaneous sensitization model of pregnant mice, the offspring of allergic mothers became tolerant to a food allergen challenge, whereas those of non-allergic mothers developed signs of systemic anaphylaxis (134). In protected offspring, FcRn was responsible for the transfer of maternal IgG IC from breast milk to neonates, induction of allergen-specific Foxp3$^+$ T_{reg} cells and long-term reduction in anaphylaxis to food allergen that persisted long after maternal-derived antibodies had disappeared. Most importantly, conditional deletion of FcRn within the APC population (*Itgax*^{cre}*Fcgrt*$^{fl/fl}$) in offspring of OVA-sensitized mothers failed to exhibit tolerance to food allergy (134). These findings illustrate that fetal and neonatal HC expressing FcRn actively promulgate tolerance to antigens comprised within maternally acquired IgG IC (**Figure 3**). Critically, such processes

extend beyond the half-life of transferred IgG and can potentially revise our concepts of passive immunity.

EMERGING ROLE OF FcRn IN CANCER

Given these observations, it is not surprising that FcRn expression in the HC promulgates antitumor activity as illustrated by the increased susceptibility of *Fcgrt*$^{-/-}$ mice to tumor development in models of colorectal cancer and lung metastasis (205, 206) (**Figure 5**). For instance, *Fcgrt*$^{-/-}$ mice exposed to the chronic carcinogen, azoxymethane and dextran sodium sulfate displayed deficient frequency and function of tissue and adjacent CD8^{+} T cells, which resulted in inability to control tumor growth in comparison to their *WT* littermates. These defects in CD8^{+} T cell numbers were dependent on the FcRn expressing CD8^{-}CD11b^{+} DC fraction, as adoptive transfer of *WT* DC conferred protection to *Fcgrt*$^{-/-}$ recipients. In addition, DC from *Fcgrt*$^{-/-}$ mice were deficient in the production of cytokines propagating cytotoxic T cell responses as mentioned above (206). Furthermore, a recent report described downregulation of FcRn expression in individuals with non-small cell lung carcinoma, which was associated with poor patient survival (224), consistent with other studies in colorectal cancer (206). More specifically, FcRn was significantly less abundant in lung tumor than non-cancerous tissue. Conversely, high FcRn expression in both cancerous and non-cancerous cells such as macrophages and DC was associated with a favorable prognosis (224).

Supporting a central role of FcRn in tumor biology is another observation reported by the Ward laboratory, but in contrast to the above it pertains to the FcRn-albumin interaction (225). The active internalization of albumin by tumor cells was recognized long before its interaction with FcRn was discovered (226). Swiercz et al. illustrated that numerous cell lines derived from breast and prostate tumors were characterized by greatly reduced FcRn expression levels. This allowed them to accumulate more albumin within cells due to reduced FcRn dependent recycling (225) (**Figure 5**). Albumin was instead diverted to and degraded in lysosomes, serving as a nutrition source for the tumor. In mouse xenograft studies, inoculation of FcRn expressing tumors resulted in more restricted growth as compared to FcRn deficient tumors which displayed accelerated tumor expansion (225). In line with this it was also recently reported that albumin conjugated to the drug doxorubicin showed better tumor inhibition efficacy in pancreatic cancer when FcRn expression was reduced. This was caused by reduced FcRn recycling, leading to increased albumin-drug catabolism (227). Overall, these results reveal that FcRn in HC and non-HC is involved in extrinsic and intrinsic control of tumor growth and that modulating FcRn function might be exploited as anti-tumor therapy.

FcRn-BASED THERAPEUTICS

Our growing understanding of FcRn's molecular structure, ligand binding properties, patterns of expression and biological functions have led to the development of therapies that aim to either exploit FcRn binding or to block it. Therefore, FcRn-based therapeutics can be subdivided in three general groups: targeted delivery, half-life extension or enhanced clearance approaches.

Targeting FcRn for Delivery of Therapeutics

There has been a great desire for enabling non-invasive delivery of therapeutics across mucosal surfaces. In addition, most communicable infections are initiated at mucosal sites, and the ensuing protective immunity involves activation of local immune cells. The role of FcRn at these locales in shuttling its ligands across the protective epithelial cell layer has thus led to the emergence of therapeutics aimed at enhancing transport of biologics across mucosal surfaces, to improve drug absorption or distribution. Indeed, fusions to IgG Fc or albumin have proven effective in pulmonary, oral, genital, and *in utero* delivery of therapeutics or vaccines.

Ye et al. showed that targeting FcRn is an effective method for transepithelial delivery of a vaccine consisting of a herpes simplex virus type-2 glycoprotein D-Fc fusion. Intranasal immunization of mice using such a construct induced efficient mucosal and systemic antibody, B and T cell immune responses, and procured stable protection for at least 6 months after vaccination (228). In another study, intranasal immunization with Fc fused human immunodeficiency virus gag protein was found to induce local and systemic immunity, as well as protection at distal mucosal sites upon vaginal challenge with a recombinant vaccinia virus expressing the human immunodeficiency virus gag protein (229). Furthermore, Pridgen et al. used Fc conjugated nanoparticles to target FcRn in the intestine for delivery of insulin across the epithelium, where it showed efficient uptake and distribution to various tissues. This delivery was dependent on FcRn as demonstrated by administration of insulin-loaded Fc nanoparticles to *WT* (33) or *Fcgrt*$^{-/-}$ mice where only the *WT* mice exhibited significant hypoglycemia (230).

Unsurprisingly, FcRn targeted therapies hold promise for fetal and neonate medicine. In one murine study, the ability of FcRn to transport IgG across the placenta was exploited to deliver an enzyme to treat lysosomal storage disease *in utero*. This was achieved through the administration of the enzyme beta-glucuronidase-Fc fusion protein to pregnant mothers which resulted in delivery of active enzyme to the fetal circulation and alleviated clinical findings associated with fetal beta-glucuronidase deficiency (231). Successful Fc-associated cargo delivery to the fetus was also recently shown for preproinsulin- and factor VIII (FVIII)-Fc fusion proteins (156, 157).

In non-human primates, FcRn expressed in the lung has been shown to enable delivery of erythropoietin (Epo) when fused to Fc and provided a distribution similar to that of Epo monomer alone delivered subcutaneously (33). The same Fc fusion Epo molecule could also be used for delivery by inhalation in humans resulting in the presence of the fusion protein constructs in serum and increase in circulating reticulocytes (232). In addition, Fc-fusion proteins of interferon-α, interferon-β and follicle-stimulating hormone can be delivered in an FcRn dependent manner via the pulmonary route (33, 232–234).

So far, the demonstration that albumin fusions can be delivered across epithelium via an FcRn/β_2m-dependent mechanism has not been established, even though albumin can be transcytosed by polarized FcRn expressing MDCK II cells in the same way as IgG (54). Albumin is present in large quantities at mucosal surfaces, similar to IgG, and in extravascular spaces (235). Further, albumin is known to be highly water-soluble and stable, and challenges related to mucosal delivery of protein-based drug formulations such as low pH, protein instability, and poor absorption may support albumin fusions as an advantageous delivery platform, as reviewed in Sleep (236). Importantly, compared to Fc as a delivery unit, albumin does not bind to classical FcγR and thus may lower the risk for unwanted immune activation. Liu et al. took advantage of albumin for efficient delivery of vaccine antigens into lymph nodes. This was achieved through attaching a fatty acid to the antigen, which bound to albumin in the circulation and further lead to lymph node accumulation (237). Interestingly, the fatty acid consisted of C18 diacyl lipid tails which bind albumin and block its interaction with FcRn (45, 54), suggesting that lymph node accumulation might have resulted from inability to engage FcRn-mediated recycling. These examples illustrate that targeting of FcRn is an efficient approach to non-invasive delivery of therapeutics and vaccines.

Half-Life Modification

Given the expanding use of monoclonal antibodies (mAb) as treatment in a range of human ailments including chronic inflammation, infections, cancer, autoimmune diseases, cardiovascular diseases and transplantation medicine, FcRn has emerged as major modifier of mAb efficacy (238, 239). This is directly related to the persistence of the therapeutic antibody in the bloodstream, which in turn can increase localization to the target site. To ensure long circulatory half-life of IgG, pH dependent binding and FcRn dependent recycling are crucial. Importantly, limited binding at neutral pH is required for proper release of IgG from cells and increasing the mAb affinity to FcRn at acidic pH correlates with half-life extension. Thus, IgG Fc engineering to optimize pH dependent binding to FcRn has been explored to tailor pharmacokinetics and increase mAb half-life (240–242). For example, the MST mutations (Met252Tyr/Ser254Thr/Thr256Glu) have enabled up to 5-fold increased persistence of IgG in humans and monkeys (240). In Phase II clinical trials the IgG^{MST} variant demonstrated half-lives of 80–120 days (242). Similarly, MN (Met428Leu/Asn434Ser) mutations, that are adjacent to the critical FcRn binding site on IgG Fc, show promise in extending IgG half-life for therapeutic antibodies (242).

Antibody engineering approaches have also been developed for more rapid degradation of target molecules, for instance toxins or inflammatory cytokines. Examples of such systems are acid-switched or calcium switched antibodies as reviewed in (243), that dissociate from their antigen at acidic pH or at lower calcium concentrations which are found in endosomal vesicles. Such antibodies will therefore bind to their target in the bloodstream and be taken up by cells. Once within the endosomal compartments, the antigen will disengage from the antibody ensuring intracellular degradation of the antigen, whereas the antibody would be protected from degradation by FcRn and recycled. In this way, the antigen circulatory half-life is limited, whereas the long half-life of the therapeutic antibody is preserved rendering it more effective even at sub-stoichiometric levels.

The ability of FcRn to prolong the half-life of its two ligands can also be exploited to extend half-lives of therapeutics by fusing a short-lived protein of interest to the Fc part of IgG or albumin. The first such fusion approved for clinical use was Etanercept (Enbrel®), which consists of the TNF receptor extracellular domain fused to the Fc part of human IgG1 (244). Etanercept competes for TNFα and TNFβ with TNF receptor and is used for treatment of rheumatoid arthritis and other forms of autoimmunity, including inflammatory bowel disease (245).

The Fc-fusion technology has also resulted in new therapeutics for treatment of hemophilia. Hemophilia A and B are X-linked bleeding disorders resulting from deficiencies of coagulation factor VIII (FVIII) and factor IX (FIX), respectively (246). Until recently, treatment required frequent injections of these factors to prevent spontaneous bleeding. A recombinant FVIII (rFVIII) fused to the Fc fragment of IgG1 (Eloctate®) was approved for clinical use in 2014. FVIII-Fc is a heterodimer that consists of one Fc chain fused to FVIII, while the other Fc is unfused, the so-called monomeric Fc-fusion (33, 247). Monomeric rFVIII-Fc allowed for less frequent administration, occurring every 4–7 days instead of every 2–3 days for rFVIII alone (248). Monomeric rFIX fused to IgG1 Fc (Alprolix®) was also approved for clinical use in 2014 and provides 3–5-fold longer half-life when compared to the rFIX alone (249).

Albutrepenonacog alfa (Idelvion®) is a fusion protein linking rFIX with albumin. A cleavable linker between rFIX and albumin is derived from the endogenous activation peptide in native FIX (250). Factor IX fused to albumin was approved for clinical use in March of 2016; this drug reduces the frequency of injections to once every 2 weeks, instead of the 2 weekly injections for rFIX alone (251–253). It was recently also shown that the albumin rFIX fusion localizes to Rab11a positive FcRn endosomes which supports the role of FcRn in promoting extended serum half-life (254). Another albumin fusion product currently approved for clinical use in the treatment of type II diabetes is Albiglutide (Eperzan®/Tanzeum®) (255). It consists of fusion of glucagon-like peptide-1, which stimulates insulin secretion by pancreatic β cells, to albumin (256). Albiglutide has a half-life of 5 days in humans (compared to minutes for unfused glucagon-like peptide-1) which allows for weekly injection regimens (255, 257). Several therapeutics based on albumin are under development or in clinical trials, and show promising results, as reviewed in (53, 236). Like IgG Fc region engineering, albumin variants with improved binding to FcRn and increased half-life are emerging. One such modified albumin, which is distinguished by a Lys-to-Pro substitution at position 573 of DIIIB, has 12-fold increased affinity for FcRn, which resulted in a significant increase in circulatory half-life in cynomolgus monkeys (258).

One major obstacle of replacement therapies is the emergence of immune responses to the therapeutic recombinant proteins in the form of neutralizing antibodies, reviewed in (259). This is exemplified by hemophilia A and B, because 40 and

4% of patients receiving rFVIII or rFIX, respectively, develop antibodies against the factor (260–262). Emerging clinical and experimental data suggest that this may less likely be the case with the Fc fusions, as the rFVIII-Fc and rFIX-Fc appear less immunogenic than the unconjugated recombinant factors alone (263–265). This is believed to occur via induction of tolerance through yet uncharacterized FcRn-dependent and -independent mechanisms (266, 267). A similar absence of immunogenicity has been described for the albumin-FIX fusion (253). Consequently, IgG Fc-, and perhaps eventually, albumin-fusion therapies might possess another unanticipated advantage by being more tolerogenic, in addition to mediating extended half-life.

Enhanced Clearance of IgG and Albumin

IgG and albumin permeate the host body and are normally innocuous. Yet in particular instances they may be harmful. This is extremely well-documented in certain autoimmune diseases, in which pathogenic self-reactive IgG antibodies play central roles (268–270). Decreasing the circulating levels of these auto-antibodies could therefore be beneficial (271, 272), and as such, the blockade of FcRn has been predicted to alleviate IgG-mediated autoimmune diseases (21, 268, 273, 274).

Several strategies have been used, including engineering antibodies with Fc regions that bind at neutral and acidic pH, anti-FcRn antibodies that block the IgG binding site, and FcRn-inhibitory peptides and small proteins (273, 275–283). Further, efficient FcRn blocking requires superior, pH independent binding to the receptor. Currently four such FcRn blocking molecules have entered clinical trials: Efgartigimod, M281, Rozanolixizumab, SYNT001, and IMVT-1401.

Efgartigimod is a human IgG1 Fc fragment that contains a constellation of "MST/HN" mutations (Met252Tyr/Ser254Thr/Thr256Glu/His433Lys/Asn434Phe) resulting in pH independent (K_D^{pH6} = 14.2 nM, $K_D^{pH7.4}$ = 320 nM), high affinity binding to hFcRn (282). Thus, upon engaging FcRn, Efgartigimod, occupies the receptor and prevents its interaction with and salvage of circulating IgGs. As this therapeutic agent possesses relatively low affinity for human FcRn at neutral pH, it is also to some degree recycled. In mice, this strategy was shown to enhance IgG clearance and to significantly reduce pathology in K/BxN arthritis and experimental autoimmune encephalomyelitis models (280, 284). In a phase I clinical trial, Efgartigimod treatment produced a rapid reduction of circulating IgG levels clearly demonstrating the effectiveness of this approach (277). At the highest administered dose of 50 mg/kg, Efgartigimod reduced all subclasses of IgG levels by approximately 50%, and multiple administration regimens (every 4 days at 10 mg/kg or 7 days at 25 mg/kg) reduced IgG levels by up to 75% (277). These effects were long lasting as antibody levels did not return to their baseline for 8 weeks post-administration. A phase II study was also recently completed where myasthenia gravis patients treated with Efgartigimod showed rapid decrease of total IgG and autoantibodies (285). Interestingly, the MST/HN mutations of IgG Fc was at the core of another approach to specifically deplete pathogenic antibodies, but it has not yet progressed to clinical trials. This strategy consists on fusing MST/HN Fc with the antigen to which the pathogenic antibody binds (286). While the antigen portion binds and traps the autoimmune antibody, the Fc portion strongly binds to FcRn, and directs the complex for rapid degradation.

Contrary to Efgartigimod, M281 is an human anti-FcRn IgG1 antibody that binds and blocks the IgG Fc binding site on FcRn (279). M281 has picomolar affinity for FcRn at both acidic (K_D^{pH6} = 43.5 pM) and neutral ($K_D^{pH7.4}$ = 28.7 pM) pH. A single administration of M281 at 60 mg/kg reduced circulating IgG levels within 2 weeks by approximately 80% from baseline. At this dose, a 20% decline from baseline was still seen 2 months after administration (279).

Another mAb designed to block IgG binding site on FcRn, Rozanolixizumab, is an IgG4P isotype that reduced IgG levels by ∼45% in humans when administered at 7 mg/kg dose (278). Although other classes of circulating Abs were not affected by Rozanolixizumab and M281 administration, a slight decrease in albumin levels was observed, possibly caused by steric hindrance at the FcRn-albumin interaction site by the bound therapeutic antibody (278, 279). Future clinical trials will show if and to which extent the depletion of circulating IgG can also affect susceptibility to infectious diseases.

Aside from blocking the IgG-FcRn interactions, there is also premise that hindering albumin binding to FcRn might also be beneficial, although the evidence for a pathological role of albumin is more ambiguous. For instance, abnormal levels of glycated albumin, observed in diabetic patients, have been associated with disease pathogenesis and tissue inflammation (287–289). Further, albumin as a carrier protein associates with many hormones, ions, metabolites and drugs, and can extend their *in vivo* half-life. Some of these molecules might be harmful at high dose, and their binding to albumin may prolong or maintain their levels in the toxic range. Acetaminophen (APAP), is a widespread analgesic that binds to albumin, and its overdose results in severe liver toxicity. We have shown that depleting albumin either via genetic deletion of FcRn or FcRn inhibition via delivery of antibodies or peptides that block the FcRn-albumin interaction, decreased APAP-mediated toxicity in mice. Although the precise mechanism of protection was not identified, it correlated with increased transport of APAP-loaded albumin into the bile and the accumulation of albumin within the hepatocyte that enhanced the intracellular albumin-mediated scavenging of reactive oxygen species (54). The therapeutic utility in blocking or enhancing albumin-FcRn interactions is less well-explored compared to IgG, largely due to inadequate understanding of albumin biology and pathology, but these studies demonstrate some potential for the albumin-docking site on FcRn as a target for future basic and translational research.

FcRn IS A RECEPTOR FOR ECHOVIRUSES

Most recently, FcRn has been identified as a receptor critical for infection with Echoviruses (290, 291) (**Box 5**), which are the leading cause of viral encephalitis and meningitis in children (294). The lack of FcRn, was thus shown to render the cells resistant to Echovirus infection while expression of

BOX 5 | Echoviruses.

Echoviruses belong to the species *Enterovirus B*, the genus *Enterovirus* of the *Picornaviridae* family. They make up the largest Enterovirus subgroup, consisting of 29 serotypes. Echoviruses are common human pathogens causing a range of illnesses such as febrile illness, but also potentially fatal conditions such as aseptic meningitis, encephalitis, paralysis and myocarditis (292, 293).

hFcRn^{TG} in mice enhanced viral infection (290). Another group illustrated binding of FcRn at neutral and acidic pH to Echovirus virions (291). Importantly, given that viral uncoating and genome release occur in acidic environment, typical of FcRn-rich endosomes, it directly implicated FcRn as Echovirus uncoating receptor. Further studies are necessary to define the precise role of this unusual Fc receptor in echovirus pathogenesis.

EMERGENCE OF FcRn FUNCTIONS DURING VERTEBRATE EVOLUTION

Lastly, it is interesting to reflect on the emergence of a receptor with an MHC-I fold, that instead of peptide presentation, binds not only IgG but also albumin in a pH-dependent fashion, and Echoviruses in a pH-independent manner. Investigation of materno-fetal transfer of antibodies have shown that aside from mammals, birds and some reptiles can transport circulating IgY, an IgG ortholog, from the mother to the yolk (295–298). In mammals and marsupials, whole genome sequence analysis of non-classical MHC-I molecules clearly identified the presence of a gene encoding the heavy chain of FcRn, while a pseudogene was detected in monotremes (299). More recently, Dijkstra et al. studying the conservation of MHC-I molecules sequence motifs, estimated that the *FCGRT* separated from the classical MHC-I lineage before the separation of monotremes and mammals around 163 million years ago (300), sometime after the divergence of amphibians and amniotes around 330 million years ago (301). Although the authors further propose that FcRn coevolved together with lactation in early mammals, this would only explain unidirectional IgG and albumin transfer into the milk and does not take into account the emergence of IgG or albumin recycling via FcRn. Further, in light of recent findings with Echoviruses, possible evolutionary pressure shaped by viral infection should also be taken into consideration. Though extremely intriguing, the study of the emergence of FcRn is still in its infancy.

CONCLUSION

FcRn controls the fate of two very distinct proteins, IgG and albumin, through a highly similar mode of binding. Despite this, FcRn's relationship with these two ligands likely involves poorly understood cooperation with other cell surface proteins and activity in different cell types that allow FcRn to manage these functionally unique proteins. Just as importantly, it is increasingly clear that FcRn functions throughout life within numerous cell types and with functional implications that are expanding into completely new areas of biology beyond what was originally envisioned. As such, the knowledge that has accumulated over the past 50 years since FcRn was imagined as a potential cellular receptor is now finally being co-opted for many exciting therapeutic purposes and in numerous areas involving drug delivery, antibody engineering, autoimmunity, cancer, and undoubtably others. It is therefore very clear that FcRn mediates much more interesting biology than its name implies.

AUTHOR CONTRIBUTIONS

MP and KS wrote the manuscript and prepared the figures. JH, JA, IS, and RB wrote and edited the manuscript.

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REVIEW
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TRIM21—From Intracellular Immunity to Therapy

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Tripartite motif containing-21 (TRIM21) is a cytosolic ubiquitin ligase and antibody receptor that provides a last line of defense against invading viruses. It does so by acting as a sensor that intercepts antibody-coated viruses that have evaded extracellular neutralization and breached the cell membrane. Upon engagement of the Fc of antibodies bound to viruses, TRIM21 triggers a coordinated effector and signaling response that prevents viral replication while at the same time inducing an anti-viral cellular state. This dual effector function is tightly regulated by auto-ubiquitination and phosphorylation. Therapeutically, TRIM21 has been shown to be detrimental in adenovirus based gene therapy, while it may be favorably utilized to prevent tau aggregation in neurodegenerative disorders. In addition, TRIM21 may synergize with the complement system to block viral replication as well as transgene expression. TRIM21 can also be utilized as a research tool to deplete specific proteins in cells and zebrafish embryos. Here, we review our current biological understanding of TRIM21 in light of its versatile functions.

Keywords: TRIM21, antibody, gene therapy, virus, infection

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INTRODUCTION

Antibodies are a crucial part of the immune response to invading viruses, and induction of neutralizing antibodies is a primary goal of vaccination (1). In addition, there is great interest in the isolation and engineering of broadly neutralizing antibodies against major human pathogens, such as human immunodeficiency virus and influenza virus for prophylactic and therapeutic uses (2–4). Antibody-mediated neutralization is generally considered to occur extracellularly due to exclusion of antibody from the cell interior by membrane compartmentalization. Extracellular neutralization has also been reported to be potentiated by engagement of Fc receptors (5, 6). However, the identification of tripartite motif containing-21 (TRIM21) as a high affinity cytosolic antibody receptor with anti-viral functions (7), has extended our understanding of the reach of antibody immunity to include the cytosol of cells.

Extracellular viral neutralization is thought to require a given level of antibody occupancy of specific epitopes to prevent entry into cells (8). However, viruses are known to display immunodominant epitopes that bias the polyclonal antibody response toward non-entry neutralizing epitopes (9–11). This implies that some viruses and bacteria have the capacity to penetrate the cell membrane and enter the cytosolic compartment even when they are opsonized with antibody. Such opportunistic pathogens are rapidly sensed by cytosolic TRIM21, which induces

a synchronized effector and signaling response. Antibody-opsonized non-enveloped viruses are rapidly targeted for degradation via the proteasome and induce an innate immune response. Bacteria in complex with antibody trigger innate immune signaling (12) and possibly killing via autophagy (13). In both cases, TRIM21 functions as a link between the intrinsic cellular self-defense system and adaptive immunity by taking advantage of the diversity of the antibody repertoire to detect invaders (14). By doing so, TRIM21 distinguishes itself from other members of the TRIM protein family with anti-viral functions as these generally recognize the invading pathogen directly (15). TRIM21 recognition is also distinct from that of other innate sensors, such as pattern recognition receptors, which detects pathogen associated molecular patterns (PAMPs) (16). Instead, TRIM21 treats the displacement of antibody from the extracellular to the intracellular environment as a danger associated molecular pattern (DAMP) (12). Viral restriction by TRIM21 may also synergize with protective anti-viral mechanisms mediated by the complement system (17, 18).

In addition to its role in intracellular defense, the implications of TRIM21 in therapy and as a research tool in cell biology are beginning to emerge. This includes identification of TRIM21 as a key player in preventing efficient adenovirus based gene delivery and vaccination (19), as well as proteasomal targeting of spreading tau protein (20). The latter promotes tau degradation instead of intracellular aggregation, which is associated with several neurodegenerative diseases. Finally, microinjection or transformation of antibody into cells can be used to direct intracellular proteins for TRIM21 mediated destruction. This technology is called TRIM-Away and is used to study protein function in cells (21, 22). These functions of TRIM21 are summarized in **Figure 1**.

In this review we discuss our current understanding of TRIM21 as a cytosolic Fc receptor that executes its effector functions alone or in synergy with the complement system. In addition, we highlight why TRIM21 should be considered in the context of disease and therapy.

TRIM21 AND ITS INTERACTION WITH ANTIBODY

TRIM21 is a multi-domain protein consisting of an N-terminal RING domain with E3 ubiquitin ligase activity, a B-box domain, a coiled-coil dimerization domain and a C-terminal PRYSPRY domain (23). The domain architecture is conserved within the TRIM protein family and it is the C-terminal PRYSPRY domain that contains the antibody binding site and thus dictates function (24). The TRIM21 PRYSPRY domain is a globular fold comprising a β-sandwich of two anti-parallel β-sheets connected by flexible loops, which are sub-divided into PRY and SPRY elements (25, 26). In solution, TRIM21 exists as a homodimer and forms a stable 1:1 complex with antibody, in which the two PRYSPRY domains bind symmetrically to the Fc (7, 25). An illustration of dimeric TRIM21 in complex with antibody is shown in **Figure 2A**.

TRIM21 is also known as Ro52 and was first identified as a major autoantigen in autoimmune diseases such as Sjogren's syndrome and systemic lupus erythematosus (SLE) (27–29). Autoantibodies generated against TRIM21 in SLE are specific for the RING and B-box domains and not the Fc binding PRYSPRY domain (30).

Interaction between TRIM21 and part of an immunoglobulin heavy chain was first reported as a false positive in a yeast two-hybrid screen (31). Since then, direct binding between *bona fide* antibodies and TRIM21 has been demonstrated and its affinity and mechanism of binding dissected by site-directed mutagenesis and binding studies combined with solving the crystal structure of the human TRIM21 PRYSPRY domain in complex with a human IgG1 Fc fragment (25). The structure confirmed that two PRYSPRY domains bind to each side of the homodimeric Fc. The TRIM21 binding site is located at the C_H2-C_H3 interface of the Fc. This is distal from the binding site for the classical Fcγ receptors and complement factor C1q (32–36), but overlaps with that of the neonatal Fc receptor (FcRn) (37, 38), as well as viral and bacterial defense proteins (39–41) (**Figure 2B**). Notably, the TRIM21-IgG interaction is largely pH-independent and unaffected by removal of the bi-antennary *N*-glycan structure attached to N297 in the Fc C_H2 domain (25, 31). It is, however, sensitive to high salt concentrations (25).

The core TRIM21-IgG1 interaction is formed between a protruding loop encompassing residues 429–436 in the Fc C_H3 domain and a deep binding pocket formed on the surface of the PRYSPRY domain (25). The apex residues H433, N434, H435, and Y436 (HNHY-motif) of the Fc loop is inserted into the PRYSPRY binding pocket. The residues form a hydrogen bond network with the base of the pocket that is protected from solvent by a shield of hydrophobic side chains. Key interacting residues in the PRYSPRY domain include D355, W381, W383, D452, F450, and W299. A detailed view of the interaction is depicted in **Figure 2C**.

The binding affinity between human IgG1 and the recombinant human PRYSPRY domain has been measured to be in the range of 150–200 nM by both isothermal titration calorimetry and surface plasmon resonance (7, 42). However, since TRIM21 is homodimeric, its functional affinity upon binding symmetrically to the IgG1 Fc is as low as 0.6 nM as measured by fluorescence anisotropy (7). This represents an increase of >300-fold compared to monomeric binding, making TRIM21 the highest affinity Fc receptor known in humans.

The TRIM21-IgG interaction is highly conserved across species, which is illustrated by the fact that both human and mouse TRIM21 efficiently bind IgG from a range of mammals (26). There is also a strict correlation between site specific mutations made in mouse TRIM21 on binding to both mouse and human IgG subclasses, which is indicative of both thermodynamic and kinetic binding conservation across species. In line with this, TRIM21 distinguishes itself from other Fc receptors in that its PRYSPRY domain does not only bind all four IgG subclasses, but also IgM and IgA. The monomeric affinity of TRIM21 PRYSPRY to IgM and IgA are much weaker, 17 and 50 μM, respectively (7, 43). This is due to large differences in

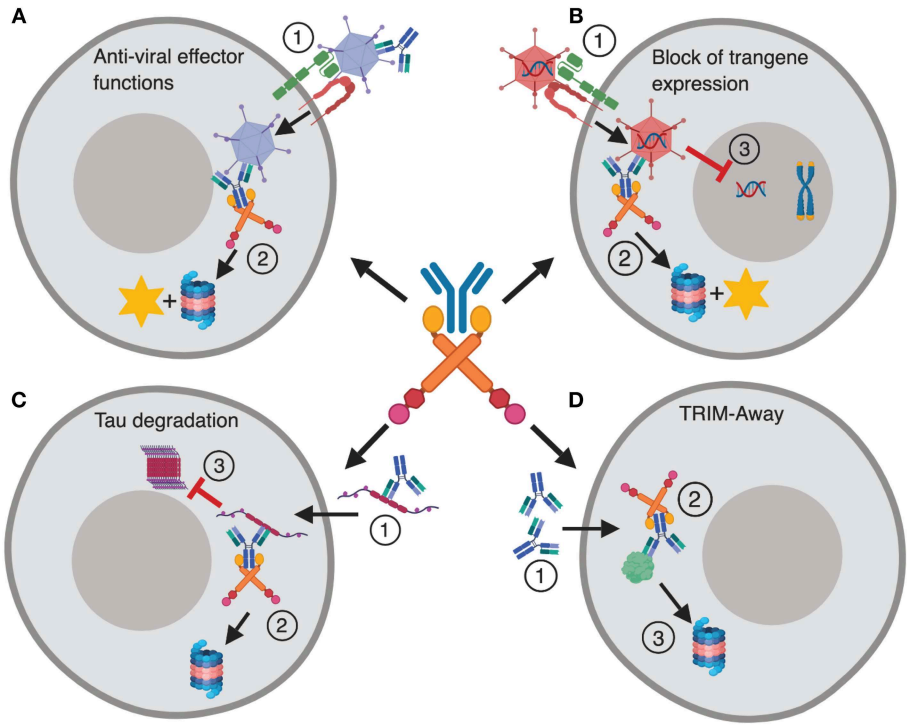

FIGURE 1 | TRIM21 in disease and therapy. **(A)** The dual effector and signaling responses mediated by TRIM21 during Ad5 infection. (**1**) Ad5 coated with antibody enters the cytosol via CAR and αvβ3/5 integrin, (**2**) and is intercepted by TRIM21 that mediates proteasomal degradation and induces innate signaling. **(B)** TRIM21 mediated block to gene therapy. (**1**) An Ad5 based gene therapy vector enters the cytosol via CAR and αvβ3/5 integrin upon which (**2**) TRIM21 targets the vector for proteasomal degradation and induces innate signaling. (**3**) This hinders nuclear delivery and transgene expression. **(C)** (**1**) Tau protein bound by antibodies enters the cytosol via endocytosis and is targeted for destruction by TRIM21 (**2**). (**3**) This prevents formation of large intracellular tau aggregates. **(D)** TRIM-Away; (**1**) Antibodies are microinjected or electroporated into cells where they bind their antigen, (**2**) TRIM21 is recruited and directs the targeted protein to proteasomal degradation. The figure was made in BioRender^{TM}.

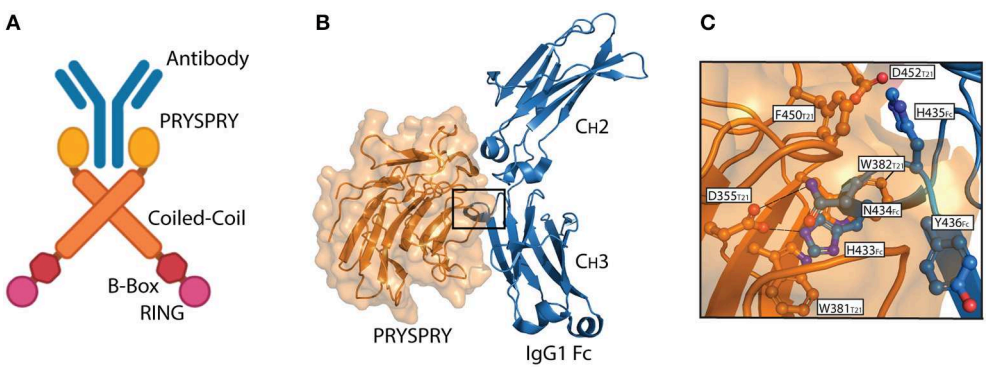

FIGURE 2 | The TRIM21-IgG interaction. **(A)** Illustration showing binding of dimeric full-length TRIM21 to antibody (blue). The RING (pink), B-Box (red), Coiled-coil (orange), and PRYSPRY (yellow) domains of TRIM21 are shown. **(B)** Structural illustration of the interaction between the globular human PRYSPRY domain (orange) of TRIM21 and human IgG1 Fc (blue) (25). Insertion of Fc loop 429–436 into the PRYSPRY binding pocked is highlighted with a square. **(C)** Close-up view showing the insertion of IgG1 Fc loop 429–436 into the hydrophobic binding pocket on TRIM21 PRYSPRY. **A** was made in BioRender^{TM} while **B,C** were made in PyMOL using PBD ID: 2IWG crystallographic data (25).

the amino acid composition of the Fc loop corresponding to the HNHY-motif in IgG, which is PNRV in IgM and PLAF in IgA. However, the loop is accommodated into the PRYSPRY binding pocket, and due to the dimeric nature of full-length TRIM21, functional affinity is likely to be in the sub-μM range (43). Both IgM and IgA have been shown to trigger TRIM21 effector functions (7, 43). Whether or not TRIM21 is able to interact with IgD or IgE has not yet been investigated, but nevertheless, it is the

only known Fc receptor that is capable of interacting with three distinct antibody isotypes.

TRIM21 COORDINATES VIRAL NEUTRALIZATION AND INNATE SIGNALING

How TRIM21 responds to antibody-coated viruses in the cytosol has been studied in detail using human adenovirus type 5 (Ad5) as a model pathogen. Upon infection of cells with Ad5-antibody complexes, TRIM21 mediates a sequential and coordinated effector and signaling response. This involves proteasomal degradation of the virus and induction of an anti-viral cellular state through activation of innate immune signaling pathways. The degradation of invading viruses by TRIM21 is termed antibody dependent intracellular neutralization (ADIN). This process occurs with rapid kinetics and can be observed with only a few antibodies per virus (44).

The dual effector response is fully dependent on the E3 ubiquitin ligase activity of TRIM21 mediated by its RING domain (45, 46). Upon engagement of antibody, TRIM21 is N-terminally monoubiquitinated by the E2 enzyme Ube2W. Then, the monoubiquitin acts as a primer for ubiquitin chain extension via a K63 linkage using the E2 enzyme complex Ube2N/Ube2V2. Depletion of cellular Ube2N leads to loss of both K63 and K48-linked ubiquitin from over-expressed TRIM21, suggesting that subsequent K48-linked ubiquitin may be incorporated, in the form of mixed or branched chains (45).

Auto-ubiquitination of TRIM21 ultimately targets the TRIM21:antibody:antigen complex to the proteasome for degradation. Here, the proteasome associated deubiquitinase Poh1 liberates the ubiquitin chains *en bloc* to induce NF-κB, AP-1 and IRF 3, 5, and 7 signaling pathways via TBK1, TAB/TAK, and NEMO (12, 46). The result is production of pro-inflammatory cytokines. The ATPase p97/valosin-containing protein (VCP), an enzyme with segregase and unfoldase activity, is needed to degrade viral particles possibly because intact capsids have to be disassembled before the individual components are degraded in the proteasome (47). Virus degradation exposes viral genomes to the cytosolic DNA and RNA sensors cGAS and RIG-1, which initiates a second wave of immune signaling in response to Ad5 or human rhinovirus 14 (HRV-14) infection, respectively (48). The dual effector functions of TRIM21 are outlined in **Figure 3**.

TRIM21 contributes to systemic protection (49) *in vivo*. This has been demonstrated for mouse-adenovirus type 1 (MAV-1) infection, both in naïve mice and mice passively transfused with MAV-1 specific anti-serum. In these experiments, TRIM21 activity prevented MAV-1 induced hemorrhagic encephalitis as mice lacking TRIM21 had higher viral loads and increased mortality compared to wild-type (WT) animals, while mice heterozygous for TRIM21 displayed an intermediate phenotype. Interestingly, the fact that naive mice demonstrate TRIM21 dependent protection suggests that the early antibody response, likely in the form of IgM, works with TRIM21 *in vivo* to prevent infection. Also, use of the fully replicative MAV-1 demonstrates that TRIM21 is effective during an active spreading infection. Together, these experiments show that TRIM21 makes a substantial contribution to systemic protection and is an important part of the humoral immune response.

Recently, the role of TRIM21 in immune activation was mapped out in detail in a genome-wide differential gene expression analysis using RNA-seq (19). The analysis revealed that immune signaling is strictly dependent on TRIM21 and its binding to antibody. In TRIM21 knockout (KO) mice gene expression in naïve or Ad5 infected animals was barely affected. Similarly, infection in the presence of the Ad5 hexon specific mouse-human chimeric rh9C12 antibody resulted in more than 700 differentially expressed genes between WT and TRIM21 KO mice (19). Furthermore, Ad5 infection in the presence of an engineered version of rh9C12, the TRIM21 non-binding IgG1-H433A (42), resulted in a similar gene expression pattern as Ad5 infection alone. Thus, both genetic knockdown of TRIM21 and abrogation of the TRIM21-IgG interaction on the protein level, prevented immune activation. Importantly, the H433A mutation specifically ablates TRIM21 binding without impairing binding to other Fc receptors or affecting the serum half-life of the antibody within the timeframe of the experiments (19, 42, 50).

The specific contribution of TRIM21 to immune signaling was determined by comparing the transcriptional changes in immune genes induced upon infection in WT vs. TRIM21 KO mice using rh9C12-WT vs. rh9C12-H433A. TRIM21 specifically induced genes related to innate and intrinsic immunity as opposed to genes associated with a strong inflammatory response such as acute phase proteins. This indicates that TRIM21 is a potent positive immune regulator that focuses the anti-viral response toward intrinsic immunity.

REGULATION OF TRIM21 IMMUNE SIGNALING

Extracellular engagement of classical transmembrane-bound Fc receptors results in intracellular activation or inhibitory signal transduction (51, 52). Their regulation is dependent upon factors such as expression level, receptor cross-linking and the ratio between activating and inhibitory receptors, which together set the activation threshold. Likewise, innate immune responses inside cells must also be tightly controlled. Members of the TRIM protein family sense ligands entering the cytosol, but it is not well-understood how they trigger signaling. One regulatory mechanism that has been proposed is that TRIM proteins are activated by higher order assembly, based on early observations of their formation of so-called cytoplasmic bodies (53). The requirement for higher order assembly to drive ubiquitination and activity was first suggested for TRIM5α (54, 55). Assembly is thought to be required to allow RING domains to dimerize, as they are normally separated from each other in TRIM dimers by a long coiled-coil. What drives TRIM assembly is less clear. Engagement of retroviral capsids by TRIM5α can induce oligomerization and hexagonal assembly via B-Box interactions between multiple TRIM5α molecules (56). Mutations that disrupt dimerization inhibit its catalytic activity (57).

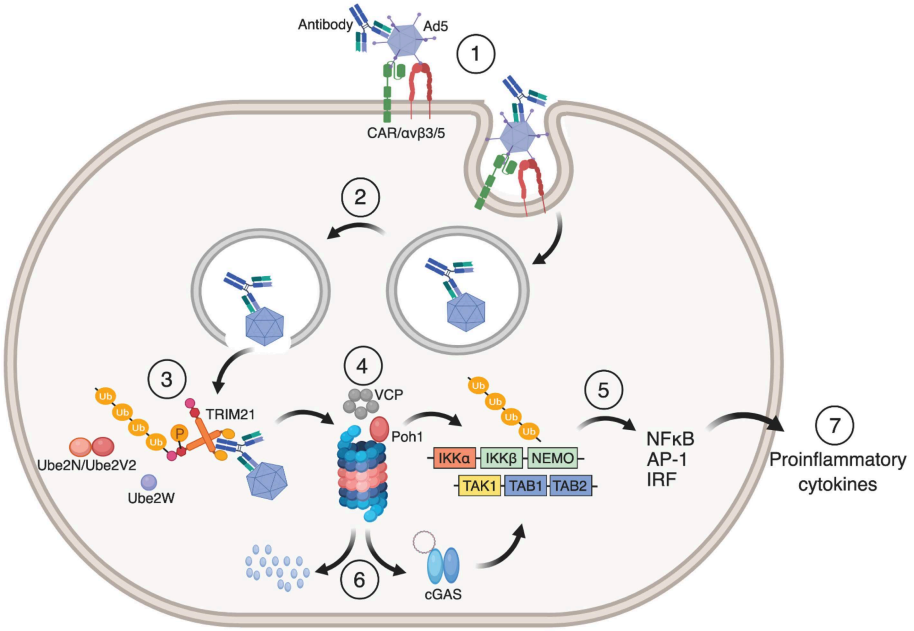

FIGURE 3 | Mechanism of TRIM21 mediated anti-viral function. (**1**) Ad5 engages CAR and αvβ3/5 integrin at the cell surface. This triggers endocytosis of the Ad5:antibody complex and loss of fiber from the Ad5 capsid. (**2**) Fiber loss exposes protein VI that lyse the endosomal membrane which allows the virus:antibody complex to escape to the cytosol. (**3**) TRIM21 binds to the Fc part of the antibody in which auto-inhibition is released by B-box phosphorylation and undergoes auto-ubiquitination by the E2 enzymes Ube2W and Ube2N/Ube2V2. (**4**) This directs the Ad5:antibody complex to VCP and the proteasome for degradation. (**5**) Liberation of K63-linked ubiquitin chains by Poh1 activates IKKα-IKKβ-NEMO and TAK-TAB1-TAB2 which in turn induces NF-κB, AP-1 and IRFs resulting in the production of pro-inflammatory cytokines and an anti-viral state. (**6**) Exposed viral genomes trigger a second wave of immune signaling via the cytosolic DNA sensor cGAS. The figure was made using BioRender^{TM}.

While RING dimerization is not required for E2 enzyme binding it is needed to form contacts with E2-charged ubiquitin. This has been demonstrated for TRIM25 as mutations at the RING dimerization interface disrupts its catalytic activity *in vitro* (58). Likewise, higher order assembly drives N-terminal K63-linked ubiquitination of TRIM5α, which is required for NF-κB activation and capsid disassembly (56). Furthermore, overexpression of TRIM5α in cells constitutively induces NF-κB activation in the absence of infection, likely due to spontaneous formation of cytoplasmic bodies (56). This in turn drives auto-ubiquitination, resulting in more rapid protein turnover by proteasomal targeting. However, such an activation strategy does not seem to be used by TRIM21 since its B-Box is auto-inhibitory (59). When TRIM21 is overexpressed in cells, no NF-κB activation or proteasomal degradation occurs in the absence of virus and antibody. Moreover, substitutions in TRIM21 corresponding to residues crucial for TRIM5α dimerization do not render TRIM21 catalytically inactive (59). These differences are observed despite the fact that the isolated RING domains of both TRIM5α and TRIM21 are highly catalytically active *in vitro*. So how is TRIM21 activation regulated?

Recent results have revealed that TRIM21 is auto-regulated via the B-Box domain, as deletion of the B-box increases the catalytic activity of its RING domain (59). This represents a novel function for the B-box domain. Structurally, this is explained by the fact that the B-box mimics and occupies the E2 ubiquitin ligase binding site on the TRIM21 RING domain, thereby preventing its auto-ubiquitination and activation of immune signaling. This was revealed by solving a crystal structure of a truncated form of TRIM21 containing only the RING and B-Box domains (59). These structural observations were confirmed by nuclear magnetic resonance (NMR) where E2 enzyme Ube2N bound to RING but not RING-B-box variants of TRIM21. Thus, for TRIM21 to respond to antibody bound virus in the cytosol a mechanism for reliving the inhibitory effect of the B-box must be in place.

The auto-inhibitory mechanism has been shown to be released by phosphorylation of a non-conserved serine residue at position 80 in the human TRIM21 RING domain by IKKβ or TBK1 kinases, driving B-box displacement from the RING (59). In line with this, stimulation of cells with poly(I:C) or infection with antibody coated Ad5 resulted in TRIM21 phosphorylation. S80 is part of a pLxSI like motif in the C-terminal end of the RING domain and located at the binding interface with the B-box. When the phospho-mimicking mutation S80E was introduced into the TRIM21 RING-B-box variant catalytic activity was restored. Cellular data support a role for phosphorylation, as infection with antibody-bound Ad5 or HRV-14 in cells expressing a S80A TRIM21 mutant did not activate NF-κB, while activation and cytokine production was restored in cells expressing

the S80E variant. Direct evidence of B-box displacement and Ube2N binding to TRIM21 have been obtained by NMR using the S80E variant (59). Interestingly, a similar regulatory mechanism exists for unrelated innate immune adaptors such as mitochondrial anti-viral signaling protein (MAVS), stimulator of interferon genes (STING) and TIR-domain-containing adapter-inducing interferon-β (TRIF), where signaling is potentiated by serine phosphorylation of a similar pLxSI motif (60).

Strikingly, TRIM21 dependent Ad5 neutralization proceeds independently of the S80 phosphorylation state, suggesting that signaling has a higher activation threshold than neutralization (59). This is an important observation since both the neutralization and signaling arms of TRIM21 require ubiquitination, which suggests that neutralization can proceed at a basal level without innate signaling being triggered. This finding is consistent with earlier studies showing that while neutralization is efficient using engineered rh9C12 IgG1 variants with up to 100-fold reduced binding for TRIM21, NF-κB induction is ablated or severely diminished (42, 44). Furthermore, design of rh9C12 IgG1 variants with differences in *on-* and *off-* rate kinetics demonstrated that while slower *off*-rates generally correlate with efficient TRIM21 activity, its signaling function was abrogated faster by a reduction in *off*-rate compared to its neutralization function (61). This may allow for a low level of TRIM21 activity without inducing an anti-viral state, which might be important for clearing low levels of free antibody displaced into the cytosol.

An important question that remains to be answered is what role TRIM21 effector functions play in different cell types. Recently, Ad5 in complex with an Fc-engineered IgG1 rh9C12 variant with 100-fold improved binding to the TRIM21 PRYSPRY domain was reported to up-regulate the co-stimulatory molecules CD80, CD83, CD86, and HLA-DR as well as increase the production of pro-inflammatory cytokines by monocyte derived dendritic cells (62). This translated into enhanced cross-priming and activation of $CD8^+$ T cells at high multiplicity of infection. In contrast, the ADIN activity was found to be unaffected. Although the exact intracellular mechanism responsible for T cell cross-priming remains to be determined, the data suggest that artificial antibodies bound to adenovirus increase the ability of dendritic cells to activate of $CD8^+$ T cells, highlighting the usefulness of Fc-engineering.

Taken together, although the effector and signaling functions of TRIM21 are synchronized, they respond in a distinct and separable manner to antibody engagement, antigen binding and auto-ubiquitination. The more stringent requirement for activation of immune signaling compared to neutralization serves to preserve an important hallmark of immunity, which is to elicit a balanced and proportionate response. If this regulation fails, the consequence of constitutive TRIM21 activation would likely be to trigger chronic inflammation. This has been demonstrated in lysosome-maturation impaired macrophages from lupus-prone mice, in which leakage of IgG containing immune complexes from the endosomal pathway into the cytosol triggers a TRIM21 dependent inflammatory response in the absence of infection (63).

SUSCEPTIBLE TARGETS FOR TRIM21

The anti-viral function of TRIM21 has been studied using several viruses including Ad5, MAV-1 and human rhinovirus 14 (HRV-14) (7, 48, 49). In addition, it has been shown that porcine TRIM21 is able to restrict Foot and mouth disease virus (64). As the requirement for TRIM21 activation is entry of an antibody-coated particle into the cytosol, it is believed that enveloped viruses are not targeted by the receptor since they leave their membrane along with bound antibodies at the cell surface upon fusion with the plasma membrane. This has been demonstrated for human respiratory syncytial virus (RSV) bound by the therapeutic monoclonal IgG1 antibody palivizumab (Synagis®) (12). Other non-enveloped viruses, such as HRV-2, form a pore in the endosomal membrane through which their genomes are injected so that its capsid and any attached antibody cannot be sensed by TRIM21 (48). The paratope of the antibodies as well as the epitope on the virus may also determine whether or not it can be targeted by TRIM21. In case of Ad5, the most studied virus in this context, its capsid fiber protein is shed during endocytosis via αvβ3/5 integrin and the coxsackie and adenovirus receptor (CAR) and as such does not follow the rest of the viral particle into the cytosol (65). Therefore, a monoclonal Ad5 fiber specific antibody poorly recruits TRIM21 (19). However, most of the antibodies in polyclonal sera are not entry blocking and target epitopes on the immunodominant major capsid protein hexon (10, 19, 66–69). Interestingly, TRIM21 sensing is not limited to viral infection, as signaling may also be triggered in response to antibody-coated bacteria, such as *Salmonella enterica* (12). In addition, non-pathogenic complexes such as host-derived proteins bound by antibody or antibody-coated beads are also targeted by TRIM21 and activate its dual effector functions (20, 47).

IMPLICATIONS FOR THERAPY

Adenoviruses are major targets for TRIM21. In gene therapy, the objective is to deliver gene variants or genes encoding vaccine epitopes for expression in host target cells (70). Adenoviruses, and in particular Ad5, is frequently used as a vehicle for these transgenes (71). However, since adenoviruses are common pathogens in humans, pre-existing antibody immunity against the virus severely limits its use as it often prevents expression of the transgene (72). Notably, normal human serum may contain as much as 100 μg/ml of Ad5 specific IgG (18). Thus, Ad5 based gene delivery vehicles will be coated with antibodies after intravenous injection (i.v.), which may explain why $CD8^+$ T-cell responses against Ad5 delivered vaccine antigens are poorly induced (72–75). However, finding a biological explanation for why the presence of antibodies blocks gene expression has been a puzzle because they do not prevent delivery of the transgene into the cytoplasmic space (73).

Recently, a substantial proportion of this antibody inhibition was demonstrated to be mediated by TRIM21 (19, 76). When naive mice were infected with Ad5 carrying a luciferase encoding transgene in the presence of the rh9C12 IgG1 antibody, expression of the transgene in the liver was reduced by 1000-fold

compared to when the virus was administered alone. Transgene expression was not affected by antibody in TRIM21 KO mice, or in WT mice using the TRIM21 non-binding mutant rh9C12-H433A. This TRIM21 dependent block to transgene expression was also observed using polyclonal mouse serum raised against Ad5. Notably, potential re-routing of virus via classical FcγR mediated uptake was not of significant importance, as infection in the presence of a rh9C12 IgG1 variant that does not bind FcγRs (L234A/L235A) did not affect the block to transgene expression (19).

Further, impaired $CD8^+$ T-cell responses against the SIINFEKL epitope (SL8) of ovalbumin (OVA) were demonstrated to be dependent on TRIM21 when mice were vaccinated with Ad5 carrying the OVA transgene in the presence of antibody (19). This resulted in reduced antiviral immunity, which was established using an engineered influenza virus strain (H1N1) carrying the SL8 peptide in its neuramidase stalk as well as reduced anti-tumor immunity as shown by challenge of Ad-OVA immunized animals with the OVA secreting fibrosarcoma cell line MCA101.

In addition to blocking transgene expression, induction of unwanted innate immune signaling may occur upon administration of adenovirus based gene delivery vectors (73, 77, 78). This may be related to the activity of TRIM21. Pre-clinically, this is supported by studies in mice infected with Ad5 in the presence of rh9C12 IgG1, where amplified transcription levels of pro-inflammatory NF-κB inducible cytokines, type-1 IFN and IFN stimulated genes (ISGs) were measured in the liver. The response was comparable to that of CpG, indicating biologically relevant induction levels. Clinical gene therapy trials indicate that pre-existing immunity to Ad5 vectors can be circumvented by intramuscular or intranasal delivery (79, 80). When Ad5 vector was given intranasally TRIM21 did not block OVA transgene delivery as potently as seen for i.v. delivery. However, this was accompanied by a 10-fold weaker OVA-specific $CD8^+$ T-cell response (19). Insights into how blockade of transgene expression delivered by adenovirus vectors is mediated opens up for development of strategies to circumvent targeting of gene therapy vectors to TRIM21. Given the reduced T-cell response associated with alternative delivery routes, a vector shielding strategy to avoid pre-existing immunity may be the most advantageous approach.

While TRIM21 negatively impacts gene therapy, the activity of the receptor may be favorably utilized for other therapeutic applications. One interesting area is neurodegenerative disorders, such as Alzheimer's disease, which are characterized by aggregation of the intracellular microtubule-associated tau protein (20). Intracellular tau aggregates are generated from extracellular tau and can spread between cells (81, 82). Administration of tau specific antibody is known to reduce tau induced pathology in mice (83–86), but the underlying mechanism has not been elucidated yet. Interestingly, TRIM21 has been shown to inhibit intracellular tau aggregation in a newly established *in vitro* tau seeding assay that recapitulates tau aggregation in diseased brains (20). Interception of extracellular tau by anti-tau antibody in the extracellular environment during cell-to-cell transfer resulted in TRIM21 dependent proteasomal degradation. As tau aggregates fast after cytoplasmic entry, the rapid recruitment of TRIM21 to incoming antibody:tau complexes are likely crucial for prevention. This discovery may foster development of new therapeutic approaches where delivery strategies for antibodies through the blood-brain barrier could be combined with targeting of extracellular tau to TRIM21 (20, 87, 88).

TRIM-AWAY

The ability of TRIM21 to target cytosolic proteins tagged by antibody for degradation can be utilized in research. This has recently been demonstrated by a technology that has been coined TRIM-Away (21). The method relies on introducing antibody, specific for an intracellular protein of interest, into single cells by microinjection or into cell cultures by electroporation (21, 22). The principle has been exemplified by microinjection of anti-GFP antibody into the human cell line NIH 3T3 over-expressing free GFP, which led to degradation of GFP within a few minutes (21). Likewise, when GFP was fused to a lipid or endogenous proteins and localized to distinct places within primary mouse oocytes, such as the plasma membrane, endosomal membranes and the nucleus, GFP was efficiently targeted by TRIM21. The depletion of nuclear proteins was dependent upon antibody access to the nucleus, and in non-dividing cells did not take place. One approach to solve this problem is to use alternative, smaller, antibody formats, for instance an Fc fragment fused to an anti-GFP nanobody that facilitated efficient nuclear transport and target degradation (21). The robustness of the method has been further studied in mouse oocytes, where targeting of the nuclear Eg5 protein resulted in rapid degradation that prevented bipolar mitotic spindle formation (21). Yet another example is targeted depletion of the long-lived Rec8 protein in mouse eggs that led to separation of sister chromatids. Importantly, this latter example addresses a major challenge, namely that long-lived proteins such as Rec8 cannot be effectively depleted using standardized DNA or RNA based methods due to their very slow turnover rate in cells (89, 90). Finally, TRIM-Away allows the depletion of specific proteins in primary human cells. This was demonstrated by depleting IKK and NLRP3 in primary human macrophages. Depletion of NLRP3 was also shown to functionally alter the macrophages, making them resistant to pyroptosis and reducing their release of potent inflammatory cytokine IL-1β (21, 91). TRIM-Away has further been used to demonstrate involvement of SNAP23 in meiotic arrest and regulation of exocytosis in developing mouse eggs (92). The usefulness of TRIM-Away to manipulate primary cells is highlighted by the fact that other approaches, such as transfection or transduction of RNA or DNA, are inefficient and stimulate innate signaling (93, 94).

The duration of knockdown by TRIM-Away correlates with the amount of antibody and TRIM21 present (21). TRIM21 is saturable when cells are exposed to high viral loads in complex with antibody (44), which is explained by its expression level, but also in part by the fact that TRIM21 is degraded together with the antibody bound target. Despite TRIM21 being IFN-inducible, which is likely crucial to sustain its

activity during an ongoing infection, the expression level of TRIM21 is in many cases a limiting factor for the TRIM-Away strategy. This is especially true when abundant cellular proteins are targeted but can be overcome by over-expression or co-administration of recombinant TRIM21 during microinjection or electroporation. Importantly, over-expression of TRIM21 does not alter the cellular transcriptome, cell phenotype or endogenous levels of other proposed TRIM21 ligands (21). When the centrosome protein percentrin was targeted in NIH 3T3 cells over-expressing TRIM21, there was efficient depletion, and percentrin failed to localize with its nuclear interaction partner Cdk5rap2. In addition, the method has been used to either activate or inhibit signal transduction pathways by targeting its different components (21). Recently, the TRIM-Away method was also reported to be an attractive tool to degrade or delay expression of proteins in zebra-fish embryos, thus enabling investigation of how specific proteins affect embryonic developmental processes (95).

A major advantage of TRIM-Away is that it is rapid and allows for monitoring of phenotypic changes in cells while at the same time limiting the appearance of compensatory mechanisms (21). On the other hand, it relies on the use of antibodies that are highly specific and do not cross-react with other intracellular proteins. A potential issue that should be taken into consideration is the possibility that targeting of one cellular protein may result in the concomitant depletion of strong interaction partners. However, such knock-on effects are also likely to result from reduced expression of the target via siRNA or shRNA or gene KO via CRISPR Cas9.

TRIM21 SYNERGY WITH COMPLEMENT

The immune system orchestrates a range of effector mechanisms to protect against infection. In humans, the complement system consists of more than 20 proteins that label pathogens for destruction via the classical, mannose lectin or alternative pathways (96, 97). After labeling, viruses may still enter endosomal compartments as well as the cytosol. As such, complement may synergize with TRIM21 in different ways to prevent cellular infection of non-enveloped viruses. These mechanisms are illustrated in **Figure 4**. This was first demonstrated by C3 deposition on the virus surface via the alternative complement pathway in a factor B and D dependent manner (17). C3 deposition triggers a dual effector response similar to that mediated by TRIM21 in the cytosol. C3 coated Ad5 is targeted to the proteasome in a VCP dependent manner where it is degraded and also induces innate signaling via NF-κB, AP-1 and IRF in non-hematopoietic cells. Further, C3 mediated immune activation may synergize with TRIM21 and antibody dependent signaling (7, 12). The efficacy of C3-dependent signaling varies among different viruses such as Ad5, HRV-14 and poliovirus P2, in which the strength of the response correlated with the degree of endosomal lysis and escape of intact C3 bound capsids into the cytosol (17). Again, enveloped viruses, such as RSV, do not efficiently trigger the cytosolic effector response.

The intracellular receptor for C3 has not yet been identified, but is likely to involve an IFN inducible gene, as C3 dependent neutralization of Ad5 is potentiated by IFN stimulation (17). The C3-dependent signaling response is dependent on MAVS and proceeds through the TNF receptor-associated (TRAF) pathway. MAVS is not required for C3 dependent neutralization which suggests that initial C3 sensing occurs via an upstream component.

While no antagonistic mechanism has been identified for TRIM21, certain viruses, such as HRV, produce cytosolic 3C proteases that cleave C3 as a protective mechanism. This has been demonstrated for HRV-14, where the 3C protease disables both signaling and degradation in the cytosol of non-hematopoietic cells, a phenotype that could be reversed by the protease inhibitor rupintrivir (17). The fact that TRIM21 uses antibody to sense pathogens, as opposed to direct deposition of C3, suggests that it is more difficult for the virus to directly antagonize it. On the other hand, some non-enveloped viruses inject their viral genome into the cytosol via pores formed in endosomal membranes, which separates antibody from TRIM21 and as such can be considered a viral protective strategy (98).

In addition to direct deposition of C3 on viral capsids via the alternative pathway, antibody opsonized pathogens may recruit the C1 complex ($C1qC1r_2C1rs_2$) to initiate the classical complement pathway. Recruitment of C1 results in cleavage of C4 followed by association of C4b with C2a to form the C3 convertase that again cleaves C3 leading to C3b opsonization and downstream formation of the membrane attack complex (MAC) (96, 97). Recently, neutralization of Ad5 was shown to occur not only via the antibody-TRIM21 axis, but also via the classical complement pathway (18). This neutralization mechanism depends on binding of C1 to antibody-opsonized Ad5 and subsequent C4b deposition on the virus, which occurs independently of other complement components.

Ad5 infection is a stepwise process that progresses from binding to the host cell receptors to endocytosis and lysis of the endosomal membrane (99–105). Ad5 engages two host cell receptors, CAR and αvβ3/5 integrin, and these binding events provide mechanical cues for the virus to commence its stepwise uncoating process. This results in loss of the fiber protein and exposure of the membrane lytic protein VI, which lyses the endosomal membrane. Upon entry into the cytosol, hexon recruits dynein and the capsid moves toward the nucleus (105–109). During this process, the capsid continues to progressively disassemble into a more meta-stable state that ultimately serves to deliver the viral genome into the nucleus. However, when C4b is deposited on an antibody coated virus, it interferes with viral disassembly in the endosome since the membrane lytic protein VI is not exposed (18). As a consequence, the virus is routed down the endo-lysosomal pathway and does not reach the cytosol. In experiments where Ad5 was opsonized with a mutant rh9C12 IgG1 variant (P329A) that fails to interact with C1, the virus readily escaped into the cytosol and is instead targeted by TRIM21. Thus, TRIM21, C1, and C4, and likely C3

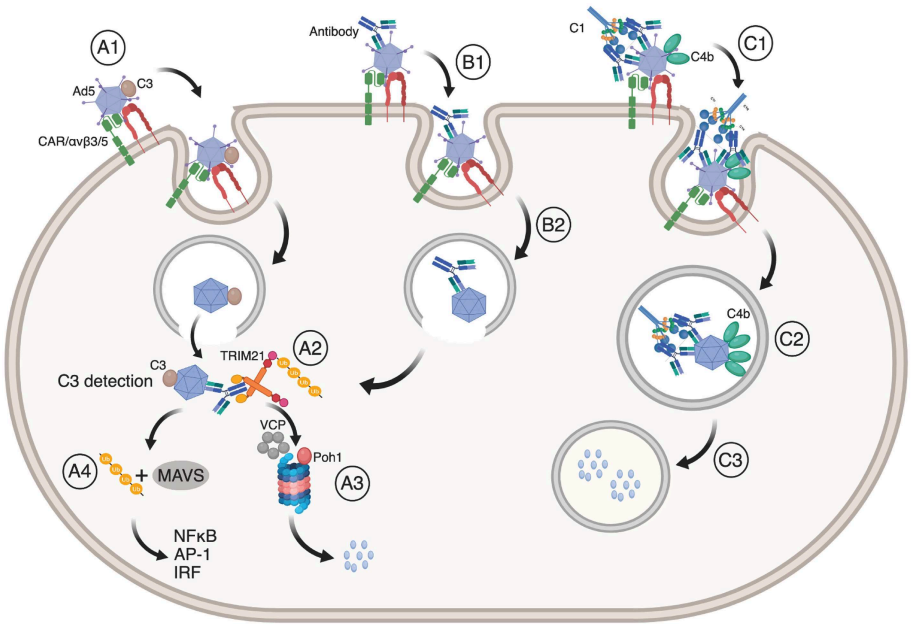

FIGURE 4 | TRIM21 synergizes with the complement system. (**A1**) Ad5 with deposited C3 enter cells via CAR and αvβ3/5 mediated endocytosis, upon which (**A2**) Ad5:C3 complexes escape into the cytosol where C3 is detected. This may occur synergistically with TRIM21 engagement (**B1, B2, A2**). In both cases, Ad5 is directed to proteasomal degradation (**A3**) followed by ubiquitin or MAVS mediated induction of NF-κB, AP-1, and IRFs (**A4**). (**C1**) Antibody coated Ad5 bound by C1 may result in C4b deposition on Ad5 prior to endocytosis via CAR and αvβ3/5 integrin. (**C2**) C4b prevents exposure of the Ad5 membrane lytic protein VI and blocks endosomal escape. Instead, Ad5 is routed into lysosomes where it is degraded (**C3**). The figure was made using BioRender^{TM}.

dependent neutralization pathways may operate synergistically during Ad5 infection (17, 18). This raises the question as to which of these neutralization mechanisms dominates under different conditions.

For C4b to be deposited on the surface of Ad5 to an extent that results in C1C4 dependent neutralization, roughly 25 rh9C12 IgG1 antibodies must bind the virus (18). The EC_{50} of C4 deposition has been determined to be 45 μg/ml, which is 10-fold lower than normal serum concentrations. As the Ad5 specific IgG titer is around 100 μg/ml in normal human serum, this strongly indicates that C4 mediated neutralization of Ad5 is likely to be a significantly contributing factor *in vivo*. This was confirmed in mice infected with Ad5 expressing a luciferase transgene where expression of the transgene was inhibited in the presence of rh9C12 IgG1, but partially restored in C4 KO mice (18). The remaining block to transgene expression was TRIM21 dependent, consistent with what is observed *in vitro*. When TRIM21 KO mice were given Ad5 in the presence of rh9C12-P329A, transgene expression was fully restored to control levels. This effect was confirmed in WT mice vaccinated with Ad5-OVA together with the double negative rh9C12 mutant IgG1-P329A/H433A that does not bind TRIM21 nor C1q, as $CD8^+$ T-cell induction was completely restored 10 days post administration. Thus, the complement system and TRIM21 may work in concert to prevent infection but also to block Ad5 based gene delivery.

ANTIBODY ENGINEERING BOOSTS GENE THERAPY

Insights into how pre-existing antibodies limit the efficacy of gene therapy, may pave the way for strategies to circumvent the problem. One conceivable strategy may be to pre-coat Ad5 vectors with an antibody engineered to ignore both C1q and TRIM21, such as P329A/H433A, or simply use a Fab fragment. Using an *in vitro* competition assay, where Ad5 infection was measured in the presence of a constant amount of rh9C12 IgG1 and increasingly higher concentrations of rh9C12-P329A/H433A or Fab, both strategies were shown to interfere with complement mediated neutralization at low concentrations (18). In contrast, much higher concentrations of the competitors were required to prevent TRIM21 mediated neutralization. This reflects that TRIM21 neutralization only requires a few antibodies bound to the virus to be effective (44), while efficient C1C4 neutralization requires higher antibody coating levels (18). When WT mice where infected with Ad5 carrying the luciferase transgene in the presence of a 1000-fold excess of a rh9C12 derived Fab fragment, a 10-fold improvement in transgene expression was observed. Importantly, the Fab pre-coating strategy also diminished the polyclonal antibody response toward the vector when administered intramuscularly to naïve mice, suggesting that this strategy could also prevent a protective antibody response during repeated vector dosing.

As hexon is the primary immunogen to which antibodies is generated, rh9C12 that binds to the apex of the hexon trimer spike (61), may be particularly well suited for such pre-coating. Indeed, shielding the Ad5 surface with a trivalent 9C12 scFv with high avidity has been used in combination with targeted designed ankyrin repeat proteins (DARPin's) binding to the fiber knob of an Ad5 vector (110). The DARPin units block CAR mediated uptake and re-targeted the vector to HER2 or EGFR positive tumor cells in mice (110, 111). This combination strategy improved the tumor to liver localization ratio of the Ad5 vector and circumvented intracellular neutralization by TRIM21, thus significantly boosting transgene expression.

CONCLUDING REMARKS

The role of TRIM21 as a high affinity cytosolic Fc receptor has grown in significance in both disease and therapy. The use of genetic KO of TRIM21, both *in vitro* and in mice, together with specific KO of the interaction on the protein level using engineered antibody variants have solidified the role of TRIM21 as a positive immune regulator whose activity is strictly dependent on detection of cytosolic antibody-virus complexes (19). It should be noted however, that TRIM21 has also been implicated in both positive and negative regulation of innate signaling independent of antibody binding (112–117). Furthermore, the identification of TRIM21 as a major player in antibody mediated block to gene therapy, together with the effect of complement factors C1 and C4, have inspired strategies to shield Ad5 gene delivery vectors from pre-existing immunity (19, 110). The finding that such shielding strategies limit immune responses to the vector should be of particular interest in cases where repeated administrations are needed.

Moreover, the remarkable ability of TRIM21 to engage three different antibody isotypes (7, 43) is in sharp contrast to other Fc receptors that are highly selective in regard to both isotype and subclass binding properties. Early primary immune responses to infections are dominated by IgM while IgG emerges later and in secondary responses upon re-infection. The IgG response is initiated by IgG3 followed by IgG1, both of which are considered anti-viral subclasses (118–121). However, how each of the four human IgG subclasses activate TRIM21 in the cytosol when bound to viruses has yet to be addressed in detail. Furthermore, since TRIM21 may synergize with the complement system, it will be important to fully understand how anti-viral immunity is orchestrated by different antibody isotypes and subclasses in the absence and presence of C1/C4.

Even though circumvention or utilization TRIM21 activity for therapeutic purposes are at the concept stage, the impact of alleviating the block to adenovirus based gene therapy or using antibody and TRIM21 to prevent intracellular tau aggregation are potentially huge. If a method to deliver antibody into the cytosol of specific cells *in vivo* with sufficient efficacy was to be developed, one could even imagine that the TRIM-Away technology could be exploited therapeutically to specifically degrade intracellular disease associated proteins (21).

Furthermore, TRIM21 function has been extensively studied using Ad5 as a model pathogen. While adenovirus infection in healthy individuals is generally benign, it may cause severe complications and even death in immunocompromised or immunosuppressed patients (122). For example, aggressive adenovirus infections in the human eye, known as *epidermic keratoconjunctivitis*, may lead to loss of vision to which there are no available treatment options (123). Whether adenovirus infections in the eye may benefit from TRIM21 and antibody therapy remains to be addressed, however, injection of antibodies into the vitreous is frequently used in the clinic to treat eye diseases such as age-related macular edema (124, 125). While activation of a strong inflammatory response may not be beneficial in a treatment setting, using engineered antibodies with reduced affinity for TRIM21 or altered antigen binding kinetics could be attractive to reduce inflammation without compromising virus neutralization (42, 61).

The ability of TRIM21 to take advantage of the antibody repertoire and orchestrate a potent intracellular immune response to invading pathogens provides non-hematopoietic cells the means to actively protect themselves even after a virus have entered the cytosol. However, as TRIM21 is also expressed by professional immune cells, its role in these cell types and how it affects antigen processing and presentation in the context of other antibody binding receptors will be important to address.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Roles of Host and Viral Antibody Fc Receptors in Herpes Simplex Virus (HSV) and Human Cytomegalovirus (HCMV) Infections and Immunity

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Herpesvirus infections are a leading cause of neurodevelopmental delay in newborns and end-organ disease in immunocompromised patients. One leading strategy to reduce the disease burden of herpesvirus infections such as herpes simplex virus (HSV) and human cytomegalovirus (HCMV) is to prevent primary acquisition by vaccination, yet vaccine development remains hampered by limited understanding of immune correlates of protection against infection. Traditionally, vaccine development has aimed to increase antibody titers with neutralizing function, which involves the direct binding of antibodies to viral particles. However, recent research has explored the numerous other responses that can be mediated by engagement of the antibody constant region (Fc) with Fc receptors (FcR) present on immune cells or with complement molecules. These functions include antiviral responses such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Uniquely, herpesviruses encode FcR that can act as distractor receptors for host antiviral IgG, thus enabling viral evasion of host defenses. This review focuses on the relative roles of neutralizing and non-neutralizing functions antibodies that target herpesvirus antigens for HSV and HCMV, as well as the roles of Fc-FcR interactions for both host defenses and viral escape.

Keywords: HSV, HCMV, herpes simplex virus, cytomegalovirus, FcR, Fc receptor, non-neutralizing antibodies, neutralizing antibodies

Herpesvirus infections are the leading cause of infectious brain damage in infants and a leading source of morbidity and mortality in immunosuppressed individuals. Neonatal herpes simplex virus (HSV) has 50% mortality in neonates who develop disseminated disease, even among those who receive appropriate antiviral therapy (1), and congenital human cytomegalovirus (HCMV) is the most common infectious cause of sensorineural hearing loss worldwide (2). In immunocompromised patients, HSV and HCMV infection can both cause severe end-organ disease. HSV-2 causes severe, sometimes refractory disease including orofacial and genital lesions in patients with HIV/AIDS and other immunocompromising conditions (3), and HCMV is a major infectious cause of morbidity and mortality in immunocompromised patients, such as recipients of allogeneic hematopoietic stem cell transplants (4). One strategy to reduce disease burden is to prevent primary acquisition or viral reactivation by vaccination. In fact, a vaccine to prevent HCMV

has been designated a "Tier I priority" by the Institute of Medicine since 2000 (5). Yet despite major advancements in research and multiple clinical trials of HSV and HCMV vaccines over the last 20 years, development of efficacious vaccines remains elusive. These challenges may be due in part to a limited understanding of the immune correlates of protection against viral infection, as well as complex mechanisms of herpesvirus evasion of these immune responses.

Traditionally, vaccine developments for HSV and HCMV have predominantly focused on the generation of neutralizing responses to prevent primary acquisition. Neutralization occurs upon direct binding of antibodies to viral antigens by their antibody binding (Fab) regions and can often be mediated in the absence of the antibody constant (Fc) region, as in the case of isolated F(ab) or F(ab)'2 fragments which are enzyme-cleaved immunoglobulin G (IgG) that lack the Fc portions. Thus, neutralization is generally achieved by antibody masking of target cell receptor binding sites or inhibition of conformational change in viral spike proteins required for fusion between the viral lipid envelope and cellular plasma membrane (6). However, the results of animal vaccine studies and recent clinical trials of HSV and HCMV vaccines have suggested that neutralization may be only one of several antibody functions that protect against HSV and HCMV infections, respectively. A previous trial of an HSV-2 subunit vaccine targeting glycoprotein D (gD), which is required for HSV entry into cells (7), elicited robust neutralization but did not confer protection against genital HSV-2 infection (8). Similarly, a subunit vaccine against HCMV glycoprotein B (gB), which is required for viral entry (9), conferred ∼50% protection in multiple phase II studies of HCMV but elicited negligible neutralizing responses against heterologous HCMV strains (10, 11).

Thus, recent vaccine efforts have aimed to measure both neutralizing and non-neutralizing antibody responses. These responses include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent complement deposition (ADCD), and antibody-dependent respiratory burst (ADRB), of which ADCC and ADCP occur upon engagement of Fc and Fc receptors (FcR) (**Figure 1**). ADCC is an adaptive immune response wherein IgG Fc-FcR engagement triggers lysis of target cells. Although ADCC activity is largely mediated by NK cells, it can also be mediated by non-NK cell populations in peripheral blood and mucosal compartments including monocytes, macrophages, and granulocytes. In ADCP, phagocytic cells such as monocytes, macrophages, neutrophils, and dendritic cells (DCs) express FcRs that enable them to efficiently uptake antibody-opsonized particles, enabling both clearance and presentation of viral antigens. The FcR most involved in the non-neutralizing antibody functions ADCC and ADCP are of the FcγR family.

FcγR is which is one of the five main FcR classes, which includes FcγR, FcεRI, FcμR, FcαRI, and FcRn, so named for the IgG that they recognize. The FcγR family is broadly categorized into three groups: FcγRI (CD64), FcγRII (CD32), and FcγRIII

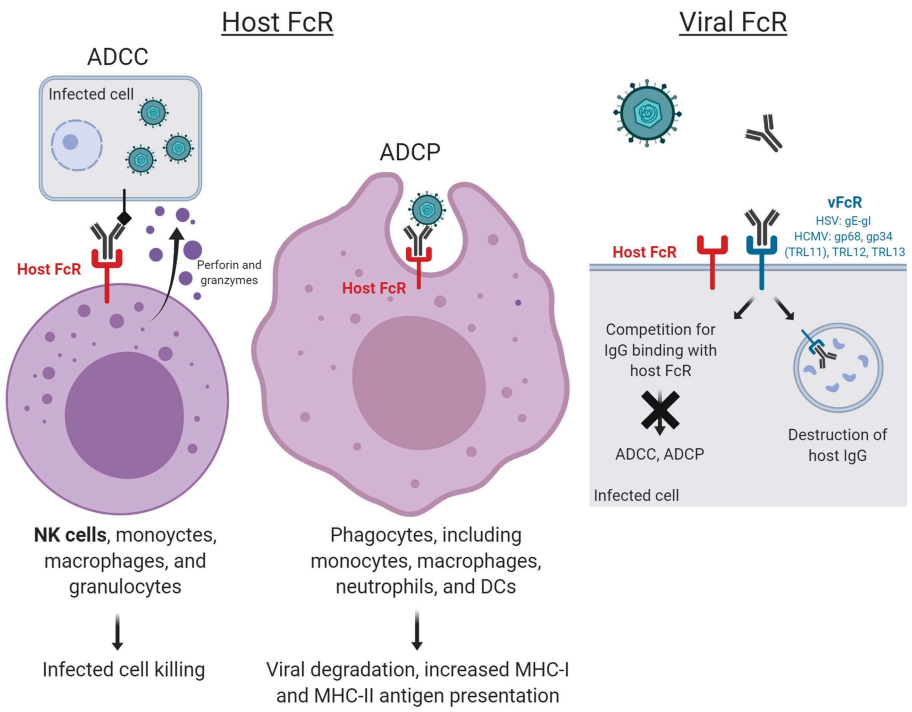

FIGURE 1 | Host and virus Fc receptor (FcR)-mediated functions. ADCC and ADCP occur upon engagement of virus-specific antibody Fc fragments to FcR, resulting in cytotoxic killing of infected cells and whole virion degradation, respectively. Herpesviruses also encode their own viral FcRs (FcRs), which recognize the Fc regions of host immunoglobulins. Mimicking host FcRs, vFcRs enable herpesviruses to reduce and evade antiviral immune responses. Figure created with BioRender.

(CD16), each of which coordinate different functions and are expressed on different cell types. The FcγRI family are high affinity (10^9/M) receptors, which can bind monomeric IgG, and are constitutively expressed on monocytes and macrophages (12). By contrast, FcγRII and FcγRIII are low-affinity (10^6/M) receptors, which bind only immune-complexed IgG, and are expressed on many hematopoietic cells. The FcγRII family is categorized into FcγRIIa, FcγRIIb, and FcγRIIc. FcγRIIa is the most widely distributed FcγR, found on neutrophils, eosinophils, B lymphocytes, platelets, mast cells, Langerhans cells, placental endothelial cells, and dendritic cells (12). In contrast to all other FcγRs which are activating, FcγRIIb is the only inhibitory FcγR, due to its unique inhibitory cytoplasmic signaling motif (13). The FcγRIII family includes two receptors: FcγRIIIa, which is expressed on monocytes, DCs, and macrophages; and FcγRIIIb, which is expressed on neutrophils, mast cells, and eosinophils. The various combinations of FcγRs play a significant role in determining an antiviral cellular response in the context of virus-specific IgG.

In humans, non-neutralizing antibody responses rely on engagement of IgG with particular FcγRs. ADCC is predominantly mediated by FcγRIIIα, FcγRI, FcγRII, FcγRIIIb, and FcαRI (CD89) (14–16). ADCP regulation is multilayered and can involve a myriad of factors, including FcR genetics, phagocyte cell type, and receptor expression pattern, tissue environment, and antibody immune complex, including specificity, isotype, subclass, and glycoforms (17). Notably, antibodies that mediate non-neutralizing functions may also mediate neutralization, and there may be not only complementary but potentially synergistic humoral effector functions for antiviral antibodies. Thus, the relative contributions of neutralizing and non-neutralizing antibody functions against HSV and HCMV for both protection and viral clearance are likely very complex.

Uniquely, herpesviruses also encode their own viral FcRs (FcRs), which recognize the Fc regions of host immunoglobulins. These vFcRs mimic host FcRs, enabling herpesviruses to reduce and evade antiviral immune responses. The elucidation of the mechanisms by which vFcRs evade host antiviral immune responses has exposed their potential as targets for novel vaccine development.

This review will discuss non-neutralizing antibody functions in HSV and HCMV, with a particular focus on functions mediated by Fc-FcR binding, as well as the role of vFcRs to mimic host FcR and to evade immune responses. An improved understanding of the distinct humoral immune correlates of protection will ultimately aid development of efficacious vaccines against herpesvirus pathogens.

FCR-MEDIATED IMMUNITY AGAINST HSV

The hallmark of HSV is its ability to establish lifelong persistent infection in sensory neurons and reactivate to cause recurrent disease or viral shedding. In HSV-infected individuals, control and clearance of the virus has been attributed to the generation of cellular immunity (18), but HSV antibodies are known to play a major role in prevention of HSV infection (19–23). In congenital HSV, maternal antibodies against HSV are known to reduce disease severity in infants (24). Women who are infected with sufficient time to transmit HSV antibodies to their infants are less likely to have infants with neonatal HSV-2 disease than women with acute HSV-2 infection at the time of childbirth (24). Thus, antibody-mediated immunity has been a central focus for HSV vaccines.

Of particular interest for HSV vaccine development are the HSV glycoproteins gD, gB, and gH/gL, which are essential for cell entry and which have been targets for multiple vaccine trials in humans (25–27). A vaccine trial of HSV-2 gD2 induced both cellular and humoral immune responses in HSV-2-seronegative patients, and despite inducing high-titer gD2-specific antibodies at levels exceeding those induced by natural infection and neutralizing antibodies, the vaccine failed to prevent HSV genital infection after 1 year of follow-up. As compared to the control group, the vaccinated demonstrated only 20% protection against genital disease (27). Surprisingly, protection against viral acquisition (with or without disease) against HSV-1 was 35% whereas there was no vaccine efficacy against HSV-2 (27). Cross-protection was expected in this trial given the high sequence homology between gD1 and gD2, yet it remained unclear what properties of the vaccine-elicited antibodies were partially protective against HSV-1 infection. In a subsequent study of the HSV-2 gD2-vaccinated women, antibody titers to HSV-2 gD2 correlated with protection against HSV-1 infection, with higher antibody concentration associated with higher efficacy, but there was no correlation between HSV-2-specific antibody titers in serum with HSV-2 protection (21). Of note, follow-up studies revealed that in sera drawn 1 month after the final dose of the HSV-2 gD2 vaccine, mean neutralizing titers to HSV-1 were 3.5 times than to HSV-2, and the mean neutralization titer against HSV-2 was 1:29, well-below that seen in natural infection (28). The results of this follow-up study may partially explain the lack of protection observed against HSV-2. Thus, although the vaccine elicited high antibody and mixed neutralizing titers to HSV but had poor efficacy against genital disease, it remains unclear if neutralization is sufficient for protection.

In addition to neutralization, recent studies have aimed to measure non-neutralizing functions of HSV-specific antibodies (**Table 1**). Mouse studies have revealed that passively infused intact HSV-specific IgG can protect against viral challenges by footpad injection, whereas F(ab')2 fragments, which can only mediate neutralization, confer only moderate protection, indicating the importance of Fc-mediated antibody functions against HSV (30). In mice, passive transfer of non-neutralizing monoclonal antibodies with *in vitro* ADCC activity protected complement-deficient mice against lethal HSV-2 challenge (29). Furthermore, in a murine challenge model of HSV-1 and HSV-2, a single-cycle HSV deleted of glycoprotein D (ΔgD-2), which is a major target of neutralizing antibodies, provided complete protection against lethal intravaginal or skin challenge, as well as rapid clearance and elimination of latent virus (39). Yet, interestingly, the vaccine-elicited antibodies had limited neutralization function and had enhanced FcR-mediated functions, namely ADCP and ADCC, as measured by activation of murine FcγRIII or FcγRIV, which of note is not expressed

TABLE 1 | Studies implicating host FcR-mediated functions in protection against HSV and HCMV infections.

Virus	Model	Functions implicated	Relevant observations	References
HSV-2	Mice	ADCC	Passively transferred non-neutralizing monoclonal antibodies with known ADCC function, measured by ^{51}Cr release, protected complement-deficient mice from HSV-2 challenge	(29)
HSV-1	Mice	FcR-mediated functions	Passive immunization with IgG, as compared to F(ab')2 treatment, reduced viral titer, and viral spread in HSV-1 challenged mice	(30)
HSV-2	Humans	ADCC	High maternal or neonatal anti-HSV ADCC antibody levels, measured by infected cell release of ^{51}Cr label, or high neonatal antiviral neutralizing levels were independently associated with an absence of disseminated HSV infection	(31)
HSV-1	Mice	ADCC	Antibodies against HSV gB or gD given with human mononuclear cells protected against lethal challenge in neonatal mice with HSV-1, and protection was associated with monoclonal ADCC activity	(32)
HSV-1	Mice	ADCC	Both neutralization and ADCC activity were independently associated with *in vivo* protection against HSV-1 challenge	(33)
HSV-2	Humans	ADCC	Among HSV-2 gB-2 and gD-2-vaccinated subjects, low ADCC responses were implicated in poor vaccine efficacy against HSV-2	(34)
HSV-2	Mice	ADCC	Antibody dependent protection against genital HSV-2 infection occurs in an Fcγ-receptor dependent mechanism	(35)
HSV-1	Mice	ADCC	HSV-1 FcγR protected the virus by blocking IgG Fc-mediated complement activation and NK cell-mediated ADCC *in vivo*.	(36)
HSV-2	Mice and guinea pigs	Not specified	Neutralization and IFNγ T cell responses did not correlate with vaccine efficacy for HSV-2 subunit vaccines containing gD or gB alone or in combination, together with CpG adjuvant	(37)
HSV-2	Mice	ADCC	The majority of sera collected from mice immunized with mature gG-2 plus CpG adjuvant showed complement-mediated cytolysis and macrophage-mediated ADCC, measured by infected cell release of ^{51}Cr label, but not neutralization	(38)
HSV-1 and HSV-2	Mice	ADCC	Single-cycle HSV ΔgD-2 vaccine conferred protection against skin challenge with clinical isolates, as well as rapid clearance and elimination of latent virus. Protection was associated with target cell killing	(39)
HSV-1 and HSV-2	Mice	ADCC, ADCP	Single-cycle HSV ΔgD-2 vaccine conferred protection against skin challenge with clinical isolates, and protection was associated with activation of HSV-specific murine FcγRIII and FcγRIV	(40)
HSV-1	Human mAbs	ADCC	mAbs derived from humans vaccinated with the HVEM binding domain of HSV-1 gD mediated neutralization and ADCC, measured by NK cell activation, and reduced ocular disease in infected mice	(41)
HSV-1 and HSV-2	Mice	ADCC, ADCP	Single-cycle HSV ΔgD-2 vaccine conferred protection against skin challenge with clinical isolates, and protection was associated with activation of HSV-specific murine FcγRIV	(42)
HCMV	Mice	Not specified	Prophylactic treatment with HCMV gB-specific neutralizing and non-neutralizing antibodies protected equally against CMV challenge. In the setting of established infection, neutralizing and non-neutralizing antibodies provided protection, with neutralizing antibodies being superior	(43)
HCMV	Humans	ADCP	An HCMV gB vaccine that afforded 50% protection in a clinical trial in post-partum women elicited limited neutralization of autologous virus and negligible neutralization of heterologous strains but robust ADCP	(10)
HCMV	Humans	ADCP	An HCMV gB vaccine that afforded partial protection in a clinical trial in transplant recipients elicited limited neutralization of autologous virus and negligible neutralization of heterologous strains but robust ADCP	(11)

gB, glycoprotein B; gD, glycoprotein D; IFNγ, interferon-gamma; gG, glycoprotein G; HSV ΔgD-2, HSV deleted of glycoprotein D.

in humans but in mice is expressed on macrophages and neutrophils (39, 40, 44). Thus, both neutralizing antibodies and ADCC appear to contribute to protection against HSV in animal models.

In human studies, non-neutralizing antibody functions are correlated with protection against infection. In follow-up studies of the HSV-2 gB2 and gD2 combination vaccine, which failed to confer protection against HSV-2 in HSV-2-seronegative women, found that the vaccine induced neutralization but had limited ADCC, as measured by target cells activation (34). A neonatal herpes study evaluated both neutralizing antibodies and ADCC titers in newborns and noted that each independently correlated with protection against neonatal HSV infection (31). These results were also recapitulated in mice (32). Previous

vaccine studies also trialed a recombinant HIV glycoprotein 120 (gp120) construct fused to the HSV-1 gD herpesvirus entry mediator binding domain (HVEM) (41), which is a cellular receptor for HSV and is expressed on lymphocytes, fibroblasts, and epithelial cells (45). Monoclonal antibodies isolated from HVEM-vaccinated individuals had both neutralization and ADCC function (45). In an *in vivo* challenge model, these human monoclonal antibodies from HVEM-vaccinated subjects protected mice from lethal infection and resulted in reduced disease burden, namely reduced ocular disease and modestly reduced virus shedding and latency after corneal inoculation with HSV-1 (45). These studies indicate the importance of Fc-mediated functions, namely ADCC, in protection against HSV in both humans and murine models and are under current investigation in HSV vaccine development.

Immunoglobulin G (IgG) genetic variations and FcγR polymorphisms are known to exert effects on ADCC functions, although this has not yet been explored extensively in the context of HSV. Previous studies have demonstrated that homozygosity for the higher-affinity allele CD16A-158V (which encodes FcγR3α) protects against symptomatic HSV-1 infection, whereas the CD32A-131H/R (which encodes FcγR2α-C) dimorphism does not (46). In a follow-up study, NK cell degranulation was consistently enhanced against opsonized HSV-1-infected targets in specifically CD16A-158V/V carriers as compared with CD16A-158F/F carriers (47). Other genetic polymorphisms for IgG and FcγR in the context of non-neutralizing antibody functions such as ADCC warrant future study.

FCR-MEDIATED IMMUNITY AGAINST HCMV

Many current vaccine strategies against HCMV infection have been designed to induce neutralizing antibody responses (48–53). However, it remains unclear whether HCMV transmission will be impacted by plasma neutralization, as reinfection occurs routinely in individuals with pre-existing immunity. *In vivo* HCMV is known to be largely cell-associated, spreading intracellularly and via cell-to-cell without diffusing into extracellular spaces as a cell-free virion (54), and clinical strains *in vitro* recapitulate this feature (54, 55). Yet, *in vitro* studies of HCMV have largely relied on laboratory strains that produce high titers of cell-free virus (56), which may be more vulnerable to neutralizing antibodies, IFN, and cellular restriction factors, as compared with virus transmitted by cell-free entry. A reconstructed wild-type HCMV strain that spread via direct cell-cell contact demonstrated that high expression of the pentameric gH/gL/gpUL128-131A complex enabled resistance to neutralizing antibodies, providing insight into potential mechanisms that facilitate the *in vivo* persistence of HCMV (57).

Although early studies had suggested that neutralizing antibodies may be protective against congenital HCMV transmission, recent randomized controlled trials in humans have indicated that neutralizing antibodies are insufficient to protect against congenital transmission, implicating a potentially important role for FcR-mediated non-neutralizing antibody responses. In a 2005, non-controlled study of HCMV congenital transmission, administration of HCMV-specific hyperimmune globulin to pregnant women with primary infection decreased the rate of mother-to-fetus transmission from 40 to 16% ($p = 0.04$), and the risk of congenital disease decreased from 50 to 3% ($p < 0.001$) (58). Subsequent non-randomized studies showed a decrease in the number of congenitally infected infants born to mothers who had been treated with hyperimmune globulin or improved outcomes in HCMV-infected infants (59–62). However, in a randomized clinical trial, the administration of polyclonal human IgG containing high titers of neutralizing antibodies failed to prevent congenital infection (63). Regarding primary infection, the most efficacious HCMV vaccine to-date was a protein subunit vaccine targeting HCMV glycoprotein B (gB), which is essential for viral entry into all cell types (9), with an MF59 adjuvant (gB/MF59), and although it achieved 50% protection against primary acquisition in multiple phase two clinical trials (64–66), sera from gB/MF59 vaccinees exhibited poor neutralization of heterologous HCMV strains (10, 11). Furthermore, a correlation between anti-gB antibody titers and protection in vaccinated transplant recipients was found to be independent of neutralization activity (11). These results suggested that the partial protection conferred by the gB/MF59 vaccine was not due to neutralizing antibodies but perhaps due to non-neutralizing antibody responses.

Follow-up studies have aimed to better characterize FcR-mediated non-neutralizing responses protective against HCMV (**Table 1**). Although the HCMV gB/MF59 vaccine did not elicit neutralizing antibodies against heterologous HCMV strains in populations of post-partum women and transplant recipients, sera from post-partum vaccinees mediated robust ADCP of both gB protein-coated beads and fluorescently-labeled whole HCMV virions by human monocytes (10, 11). Interestingly, the gB/MF59 vaccine preferentially induced high binding magnitude gB-specific responses of the IgG3 isotype (10), which is known to demonstrate high avidity for FcR on monocytes and macrophages and which has been shown to coordinated multiple antibody effector functions including ADCC and ADCP (67, 68). Vaccine-elicited antibody enhancement of phagocytosis is thought to have contributed to the partial efficacy of the HCMV gB subunit vaccine, though it remains unclear if ADCP is necessary or sufficient for protection against disease and warrants further study.

In HCMV, ADCC appears to play a role in antiviral immunity for naturally infected individuals, but its importance in protection for vaccine-elicited responses remains to be determined. Studies of pooled human IgG from naturally seropositive individuals (Cytogam) can promote antibody-mediated NK cell lysis (69), and ADCC is measurable in naturally seropositive subjects (10). However, postnatal and transplant subjects vaccinated with gB/MF59 demonstrated no substantial ADCC-promoting antibody response in *in vitro* assays with human NK cells (10, 11). In a murine model of CMV infection, prophylactic administration of HCMV gB-specific monoclonal antibodies before infection was also protective, and both neutralizing and non-neutralizing mAbs were equally effective in preventing lethal infection of immunodeficient mice (43). Thus, FcR-mediated non-neutralizing antibody functions such as ADCP and ADCC against HCMV appear to be involved

in the antiviral immune response, but their separate and overlapping contributions with neutralizing responses remain to be determined.

HSV AND HCMV VIRAL FCR IN IMMUNE EVASION

Uniquely, members of the α- and β-subfamily of *herpesviridae* establish permanent, lifelong infections in their hosts. They achieve this in part by encoding surface glycoproteins that bind to the Fc region of host IgG and facilitate evasion from the host immune response (70). HSV and HCMV encode a number of immunomodulating proteins such as decoy receptors and chemokines, which are theorized to protect against both innate and adaptive immune responses (71).

HSV-1 encodes surface glycoproteins gE and gI, which can form a complex on infected cells or on the virion surface that binds to the Fc domain of host IgG (72, 73). This complex acts as a vFcR and is associated with cell-to-cell spread of infection (72, 73). The HSV gE-gI complex is required for the binding of monomeric non-immune IgG, but HSV gE alone is sufficient for binding polymeric IgG (74). The HSV gE-gI complex is thought to facilitate degradation of antiviral host antibodies through pH-specific binding. In this process, host anti-HSV IgG antibodies participate in antibody bipolar bridging, whereby an HSV-specific host antibody simultaneously binds to the HSV gE-gI complex with its Fc region and to a specific HSV-antigen (e.g., gC or gD) with its Fab arms (75–78). At the basic pH of the cell surface, anti-HSV antibody can bind to both HSV gE-gI complex and HSV antigen, but once this antibody is endocytosed and trafficked into the late endosomes, the HSV gE-gI complex dissociates from the antibody Fc region. The host antibody bound to HSV antigen is then localized to the lysosome, where both are degraded, whereas the HSV gE-gI complex can be recycled back to the cell surface. This process of antibody bipolar bridging protects virally infected cells from antibody- and complement-dependent neutralization (78), ADCC (36), and granulocyte attachment (79), and is thus an important mechanism of host immune evasion from antibody-mediated clearance.

One novel strategy for vaccine development against HSV infection aims to prevent these viral immune evasion activities (**Figure 1**). In fact, a trivalent HSV vaccine composed of the vFcγR HSV-2 glycoproteins C, D, and E has been tested in animal challenge studies, in which the vaccine protected seronegative rhesus macaques against intravaginal challenge and seronegative guinea pigs against severe genital disease (80). These glycoproteins were selected due to the involvement of HSV-2 gC in complement cascade inhibition, thus contributing to immune evasion (81); gD in virus entry (26); and gE in blocking host IgG Fc thus also contributing to immune evasion (82). Immunogenicity data revealed that the vaccine induced plasma and mucosa neutralizing antibodies, antibodies that block gC2 and gE2 immune evasion activities, and stimulated CD4 T cell responses (80). In guinea pigs previously infected intravaginally with HSV-2, the vaccine reduced the frequency of recurrent genital lesions and the frequency and duration of vaginal shedding. These studies demonstrate the potential for vaccine candidates aimed at preventing HSV evasion from host defenses in the context of both primary infection and reactivation and require further studies in humans.

Human HCMV encodes four glycoproteins that act as vFcγR and interfere with IgG-mediated immunity against HCMV: gp68, gp34 (toll-like receptor 11/TLR11), TLR12, and TLR13 (83–85), each with a unique binding pattern to host IgG. Distinct from host FcγR, HCMV vFcγR demonstrate glycan independent binding (86), and all HCMV FcγR genes are transcribed with relatively delayed kinetics during the protracted viral replication cycle, reaching abundant protein amounts during the late phase of infection (83). HCMV gp68 and gp34 are specific for binding human IgG but do not discriminate among the IgG subclasses (87). Recent studies reported formation of antibody bipolar bridging complexes with gp68 and with gp34, and that HCMV lacking gp34 or/and gp68 elicited much stronger activation of host FcγRI, FcγRIIA, and FcγRIIIA by polyclonal HCMV-immune IgG as compared to wildtype HCMV (71). These results implicate HCMV gp34 and gp68 in evading the host FcR-mediated immune response. Unlike the HSV-1 gE-gI complexes, the gp68-Fc interaction is broadly stable across acidic and basic pHs (86), resulting in degradation of the HCMV vFcγR gp68 with the host antibody and HCMV antigen. It is clear that vFcRs are a unique viral immune evasion factor, and further investigation will be required to understand the role of these receptors in both viral pathogeneses, and as potential novel targets for vaccine development.

CONCLUSION

Herpes simplex virus (HSV) and HCMV infections are a serious cause of morbidity and mortality among infants and immunocompromised patients worldwide. There is an urgent need for efficacious vaccines against these pathogens, both to prevent primary acquisition as well as reactivation of latent virus. Historically, vaccine development has aimed to increase the titer of neutralizing antibodies against HSV or HCMV to confer protection, but recent clinical trial data and follow-up immunogenicity studies have investigated the roles of antibody Fc-mediated functions, namely ADCC and ADCP. Furthermore, herpesviruses uniquely encode vFcRs that promote destruction of antiviral host IgG and may enable immune evasion. An improved understanding of non-neutralizing antiviral immune responses and herpesvirus vFcRs may illuminate new pathways for the development of more efficacious vaccines against HSV and HCMV infections.

AUTHOR CONTRIBUTIONS

JJ wrote the majority of the manuscript. MG wrote and edited the manuscript. SP is the PI of JJ and MG. She oversaw the writing and made significant editing contributions.

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REVIEW
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Genetic Variation in Low-To-Medium-Affinity Fcγ Receptors: Functional Consequences, Disease Associations, and Opportunities for Personalized Medicine

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Fc-gamma receptors (FcγR) are the cellular receptors for Immunoglobulin G (IgG). Upon binding of complexed IgG, FcγRs can trigger various cellular immune effector functions, thereby linking the adaptive and innate immune systems. In humans, six classic FcγRs are known: one high-affinity receptor (FcγRI) and five low-to-medium-affinity FcγRs (FcγRIIA, -B and -C, FcγRIIIA and -B). In this review we describe the five genes encoding the low-to-medium -affinity FcγRs (*FCGR2A, FCGR2B, FCGR2C, FCGR3A,* and *FCGR3B)*, including well-characterized functionally relevant single nucleotide polymorphisms (SNPs), haplotypes as well as copy number variants (CNVs), which occur in distinct copy number regions across the locus. The evolution of the locus is also discussed. Importantly, we recommend a consistent nomenclature of genetic variants in the *FCGR2/3* locus. Next, we focus on the relevance of genetic variation in the *FCGR2/3* locus in auto-immune and auto-inflammatory diseases, highlighting pathophysiological insights that are informed by genetic association studies. Finally, we illustrate how specific FcγR variants relate to variation in treatment responses and prognosis amongst autoimmune diseases, cancer and transplant immunology, suggesting novel opportunities for personalized medicine.

Keywords: Fc gamma receptor (FcγR), genetic variation, autoinflammatory and autoimmune diseases, immunotherapy, mechanisms of disease

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INTRODUCTION

Fc-gamma receptors (FcγR) are cellular receptors for Immunoglobulin G (IgG) and upon binding of complexed IgG can trigger various cellular immune effector functions that can destroy and eliminate the opsonized target. These responses are only initiated when multiple IgG molecules are bound simultaneously; a single IgG does not activate FcγRs. When multiple IgG molecules are fixed close to each other, as is the case in opsonized bacteria or in an immune complex, this results in cross-linking of several FcγRs in the cell membrane, which leads to their activation. In this way, FcγRs play an important role in immunity by linking the adaptive and innate immune systems.

FcγRs are differentially expressed on a range of immune cells (**Figure 1**) (3). In humans, six classic FcγRs are known, which can be distinguished into one high-affinity receptor (FcγRI) and five low-to-medium-affinity FcγRs (FcγRIIA, -B and -C, FcγRIIIA and -B). The low-to-medium-affinity FcγRs are the focus of this review. On a functional level, most of the FcγRs are activating receptors that can induce the cellular responses mentioned above, but one, FcγRIIB, is an inhibitory receptor.

The five genes for the low-to-medium-affinity FcγRs are located in a single cluster on chromosome 1q23.3 (the *FCGR2/3* locus) and several genetic variations resulting in functional changes have been found in all of the genes in this locus. These variations are associated with auto-immune, auto-inflammatory, and infectious diseases and with efficacy of immunotherapy in cancer patients, but genetic analysis of the variants at the locus is hampered by the genetic complexity deriving from a segmental duplication, inconsistent nomenclature, and a high degree of linkage disequilibrium.

The gene encoding FcγRI, *FCGR1A*, is also located at chromosome 1 (1q21.2), and has two presumed pseudogenes (*FCGR1B* at 1p11.2 and *FCGR1C* at 1q21.1) that have stop

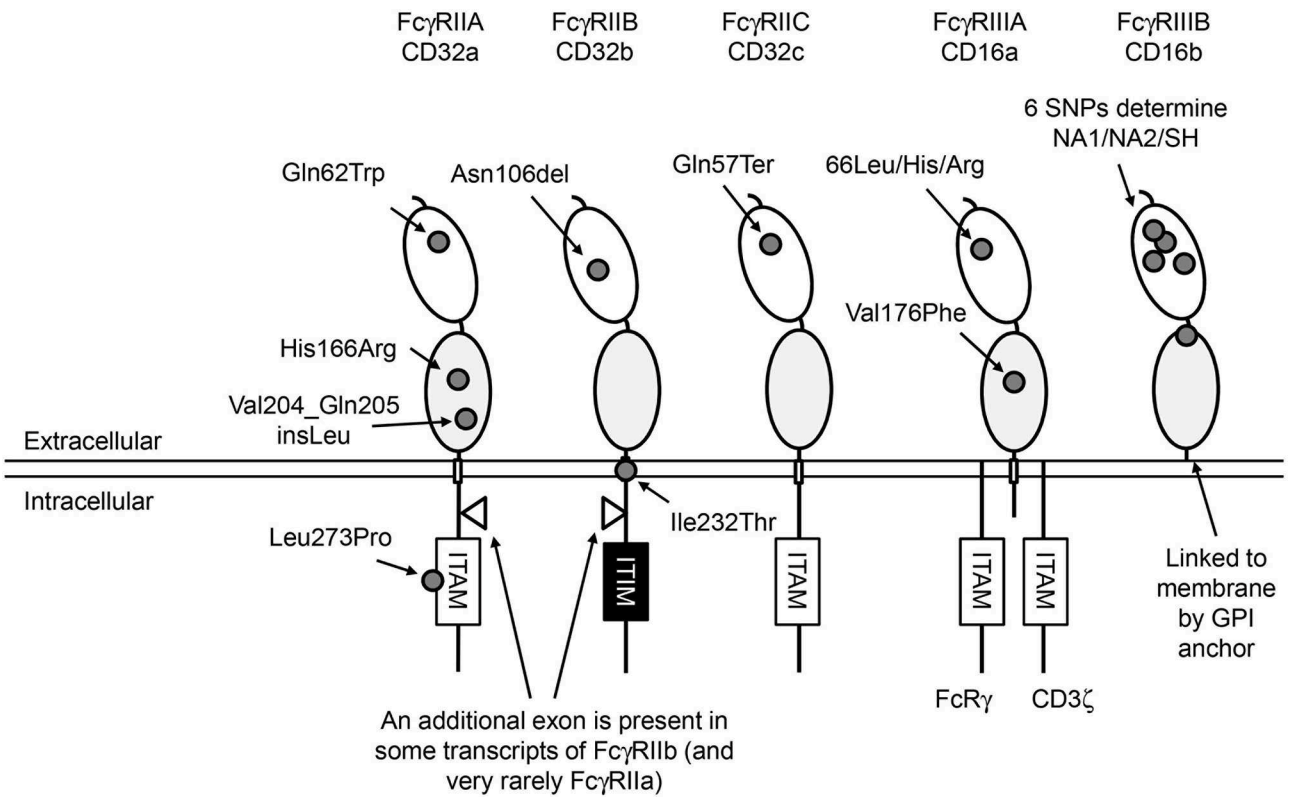

FIGURE 1 | Human FcγRs. Overview of the structure of human low-to-medium-affinity FcγRs. The oval shapes in the extracellular part of the FcγRs represent the different extracellular domains; the light gray domains are the domains where IgG molecules bind. All FcγRs except FcγRIIIB are linked to the plasma membrane by transmembrane (TM) domains indicated by small rectangles. FcγRIIIB is linked to the plasma membrane through a GPI anchor. FcγRIIIA has a small intracellular domain that associates with adaptor molecules that can initiate an intracellular signaling cascade when multiple FcγRs are cross-linked, which ultimately leads to activation of the cell on which the FcγRs are expressed. FcγRII receptors have a much larger intracellular domain, and contain a signaling motif to start this cascade in their own polypeptide chain. Signaling by activating FcγRs is mediated by immunoreceptor tyrosine-based activating motifs (ITAM) that are present either in the cytoplasmic tail of the receptor itself or in non-covalently associated signaling adaptor proteins, such as the common γ-chain (FcRγ). Aggregation of activating FcγR by binding of multivalent ligands, such as an opsonized pathogen or blood cell or an immune complex, results in the phosphorylation of ITAM tyrosine residues by Src family protein tyrosine kinases (PTKs), and ultimately leads to activation of cellular responses (1). Aggregation of the inhibitory FcγRIIB, which contains an immunoreceptor tyrosine-based *inhibitory* motif (ITIM), also results in phosphorylation of tyrosine residues by Src family PTKs. In contrast to ITAMs, phosphorylated ITIMs serve as binding sites for phosphotyrosine phosphatases (PTPs) which dephosphorylate other proteins resulting in inhibition of activating pathways (2). Approximate location of functional SNPs in the FcγRs are indicated by small gray circles, SNPs are indicated by 3-letter amino-acid codes. ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif.

codons in the third extracellular domain and theoretically cannot be expressed as transmembrane receptors (4). Recently, some functional SNPs that occur at low frequency in the population were discovered in *FCGR1A* (5, 6), but because this gene lies far outside the complex *FCGR2/3* locus and no disease associations have been described yet, these SNPs are beyond the scope of this review.

We provide an overview of the currently known genetic variation in low-to-medium-affinity FcγRs, with a focus on the genetic challenges in characterizing this locus, nomenclature of the variations, functional consequences, disease associations with specific diseases and in general, and will discuss the potential of *FCGR2/3* genotyping for personalized medicine.

LOW-TO-MEDIUM-AFFINITY FC-GAMMA RECEPTORS

IgG-FcγR interactions depend on the IgG subclass (IgG1, IgG2, IgG3, and IgG4) and IgG-Fc glycosylation structure of p.Asn297 in the IgG protein, as well as on the specific FcγR and variation within its amino acid sequence by genetic polymorphisms (7, 8). A schematic representation of the low-to-medium-affinity Fc-gamma receptors and the approximate location of the genetic variants is provided in **Figure 1**.

FcγRIIA (CD32a) consists of a single polypeptide chain which contains an immunoreceptor tyrosine-based activating motif (ITAM) in the intracellular domain. FcγRIIA is the most widely expressed isoform of FcγRII and is found on monocytes, macrophages, dendritic cells, neutrophils, and platelets. It can induce many different cellular defense mechanisms such as phagocytosis of IgG-opsonized targets, antibody-dependent cellular cytotoxicity (ADCC), production of reactive oxygen species (ROS), and cytokine production.

FcγRIIB (CD32b) is the only FcγR that results in an inhibitory signal to the cell, which is transferred by the immunoreceptor tyrosine-based inhibitory motif (ITIM) on its intracellular signaling domain. FcγRIIB is found in two isoforms deriving from two different transcripts (**Figure 1**), FcγRIIB-1 and FcγRIIB-2, with FcγRIIB-1 having an additional intracellular exon in between the transmembrane and signaling domains. FcγRIIB-1 is highly expressed on B cells, where it constitutes the only surface-expressed FcγR, and co-crosslinking of FcγRIIB-1 with the B cell receptor (BCR) inhibits activating signals induced by the BCR. Other cell types also express FcγRIIB, albeit at much lower levels, and on these cells FcγRIIB-2 is the main transcript expressed. These cells include a subset of monocytes, macrophages, and dendritic cells. Expression of FcγRIIB can also be detected on neutrophils and NK cells, but only in individuals with certain genotypes (9–11). When transfected in COS-1 cells, FcγRIIB can inhibit pro-phagocytic signals induced by activating FcγRs, balancing the immune response against IgG-opsonized targets (12), but it remains currently unknown if this mechanism is also involved in myeloid cells. Interestingly, at phagocytic cups, FcγRIIB may be relatively excluded whereas FcγRIIA is enriched, likely due to their difference in IgG affinity, which may affect the ability of FcγRIIB to exert inhibitory signals (13).

FcγRIIC (CD32c) has long been considered not to be expressed at all, as its gene (*FCGR2C*) was thought to be a pseudogene (14, 15), and therefore relatively little was known about the expression pattern of this receptor. In 1998, FcγRIIC was first found on NK cells of individuals that carry an open reading frame (ORF) of this receptor (p.57Gln, *FCGR2C*-ORF), as opposed to the majority of individuals in which this receptor is indeed a pseudogene and cannot be expressed as a result of a stop codon in exon3 (p.57Ter, *FCGR2C*-Stop) (16). Determining the cellular expression pattern of FcγRIIC has long been complicated because the extracellular domains are identical to FcγRIIB, but specific detection of FcγRIIC is possible by comparison of cellular expression between individuals that can or cannot express FcγRIIC as a result of the stop codon, detection of FCGR2C mRNA and western blots of immunoprecipitated FcγRIIC. We now know that FcγRIIC can be expressed on NK cells, neutrophils, monocytes (9), and macrophages (17). This receptor has also been reported to be expressed on B cells (18) although expression on B cells could not be reproduced in our own laboratory (17). Obviously, FcγRIIC can only be functional in individuals with an *FCGR2C*-ORF. Although expression on NK cells is relatively low, it has been shown to be capable of inducing killing of target cells in a redirected ADCC assay (19), functioning as an activating receptor.

FcγRIIIA (CD16a) has two extracellular (EC) Ig-like domains, involved in binding of IgG, a transmembrane (TM) domain and a short intracellular (IC) domain. The TM domain associates with adaptor proteins containing an immunoreceptor tyrosine-based activating motif (ITAM) to induce intracellular signaling. In monocytes and macrophages, this receptor associates with the FcRγ-chain, while in NK cells it associates with the CD3 ζ-chain (20–22). Association with these adaptor proteins is not only essential for signaling and maintaining stable expression, but also for targeting the receptor to the cell membrane (22). FcγRIIIA expressed on NK cells can induce ADCC by these cells (23), and on phagocytes it can induce phagocytosis (24). Recently, it was suggested that FcγRIIIA is also expressed in low levels on neutrophils (25), which is surprising since it was never found before and contradicts the finding that two donors completely deficient for FcγRIIIB did not show any staining on neutrophils with a MoAb (3G8) that recognizes both FcγRIIIA and FcγRIIIB (11).

FcγRIIIB (CD16b) is a GPI-anchored protein, expressed in high numbers on neutrophils, and sometimes on eosinophils. As it does not have a transmembrane domain, it cannot associate with FcRγ or the ζ-chain. FcγRIIIB is not capable of IgG-induced production of ROS (26). However, it does contribute in *in vitro* experiments to the exocytosis of neutrophil granule proteins (27) and Ca^{2+} influx (28), and may also cooperate with FcγRIIA on the same neutrophil to induce such responses (29). Because FcγRIIIB can induce these responses, FcγRIIIB has often been classified as an activating receptor, although the exact mechanism(s) by which FcγRIIIB activates cells are still unclear (30, 31). Nowadays FcγRIIIB is mainly seen as a decoy receptor (32, 33) as it has a clear role in binding to—and mediating internalization of—soluble immune complexes in neutrophils (34, 35). In fact, FcγRIIIB can also decrease

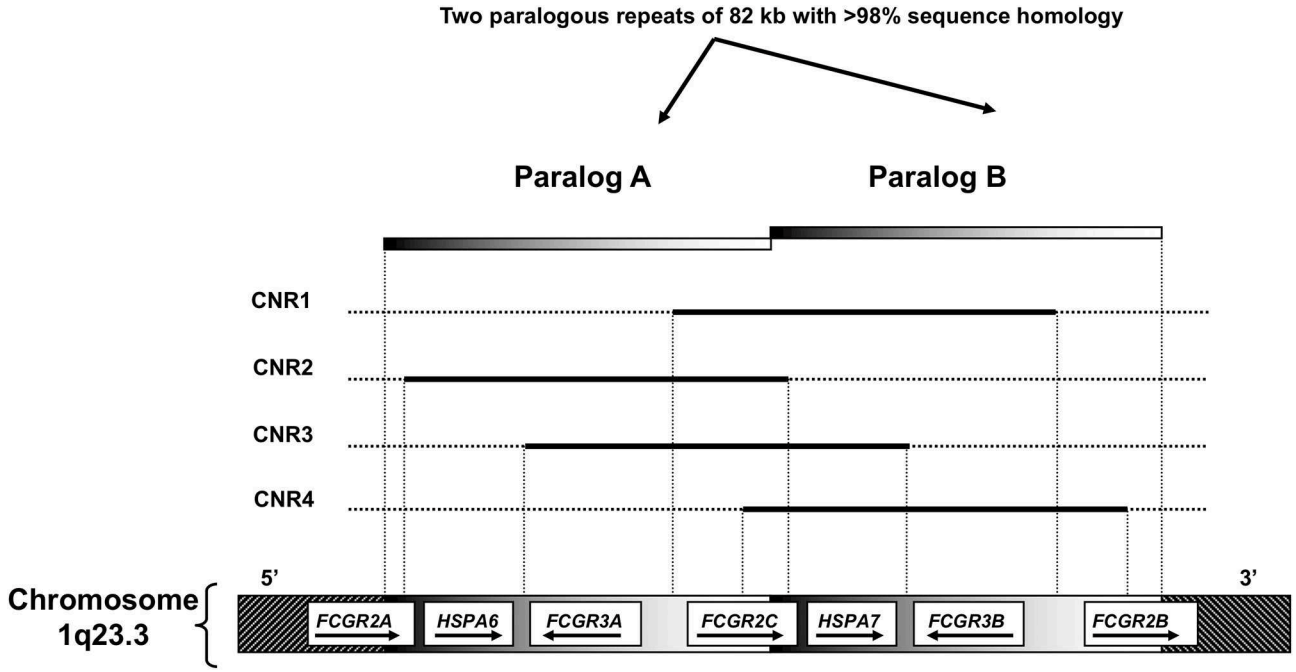

FIGURE 2 | Overview of the *FCGR2/3* locus. A Structural overview of the locus, with the orientation of the genes indicated by arrows. B Overview of copy number variation at the locus. Four combinations (copy number variable regions, CNRs) of FcγR genes have been shown to occur in duplication/deletion. Black lines indicate which genes are involved in copy number variation (CNV).

antibody-mediated trogocytosis by neutrophils (36). In FcγR-deficient mice transgenic for human FcγRIIIB, FcγRIIIB mediates the interaction of neutrophils with soluble immune complexes and render the neutrophil susceptible to tissue adhesion and capillary transmigration (37). FcγRIIIB also allows flowing neutrophils to tether to IgG and immune complexes (38).

THE *FCGR2/3* LOCUS

The low-to-medium-affinity FcγRs; FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA and FcγRIIIB are encoded respectively by *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B*. All these genes are located in a cluster at 1q23.3 in the *FCGR2/3* locus (**Figure 2**). The locus consists of two 82 kb paralogous repeats with >98% sequence homology, that were formed as the result of an unequal crossover event (15). This unequal crossover event between *FCGR2A* and *FCGR2B*, the two genes that flank the region, has led to a segmental duplication in which *FCGR2C* was formed (15), with the resulting *FCGR2C* gene being highly homologous to *FCGR2B* in the first six exons and highly homologous to *FCGR2A* in the last 2 exons. **Figure 3** provides an overview of the differences between the three *FCGR2* genes. Furthermore, the segmental duplication created the two different *FCGR3* genes, *FCGR3A* and *FCGR3B*, which are also highly homologous in sequence (**Figure 4**). The genes encoding the classic FcγRs are highly polymorphic and functionally relevant genetic variations have been described for all low-to-medium-affinity FcγRs. An overview of the functionally relevant SNPs, is given in **Table 1**, and the approximate locations within the FcγRs are shown in **Figure 1**. The functional consequences of the SNPs are discussed below.

Besides being polymorphic, some of the low-to-medium-affinity *FCGR* genes are subject to gene copy number variation (CNV). Although several large-scale studies on CNV have suggested that human *FCGR2A* and *FCGR2B* are candidate genes for CNV (95, 96), our group has shown previously that this is not the case. In fact, CNV in the *FCGR* locus is restricted to *FCGR2C*, *FCGR3A,* and *FCGR3B* (63). CNV at the *FCGR2/3* locus always occurs in distinct copy number regions (CNRs) that consist of a complete stretch of 82 kb generated by non-allelic homologous recombination (NAHR) (**Figure 2**) (56, 97, 98). The clear distinction in CNRs suggests that there must be hotspots for NAHR breakpoints. Breakpoints for the most common CNR1 have been studied in more detail and it appears that these consist of several different breakpoints (81, 97). Exact localization of breakpoints for CNR1 may not always be possible because many potential breakpoints for CNR1 lie within a 24.5 kb block in which no genuine paralogous sequence variants (PSVs) are present (i.e., no clear distinction between paralog A and paralog B can be made and therefore no absolute conclusion on the position of a breakpoint) (99). This block comprises of both the 3′ end of the intergenic region between *FCGR3B* and *FCGR2B* and the first exons of *FCGR2B*. Breakpoints for the rare CNR4 (which have a breakpoint distal of exon3 of *FCGR2B*) can also lie within this 24.5 kb block. This 24.5 kb "block" may however be a result of a combination of different (smaller) gene conversion events, and Rahbari et al. later showed that it was possible to define breakpoints even within this 24.5 kb block (81).

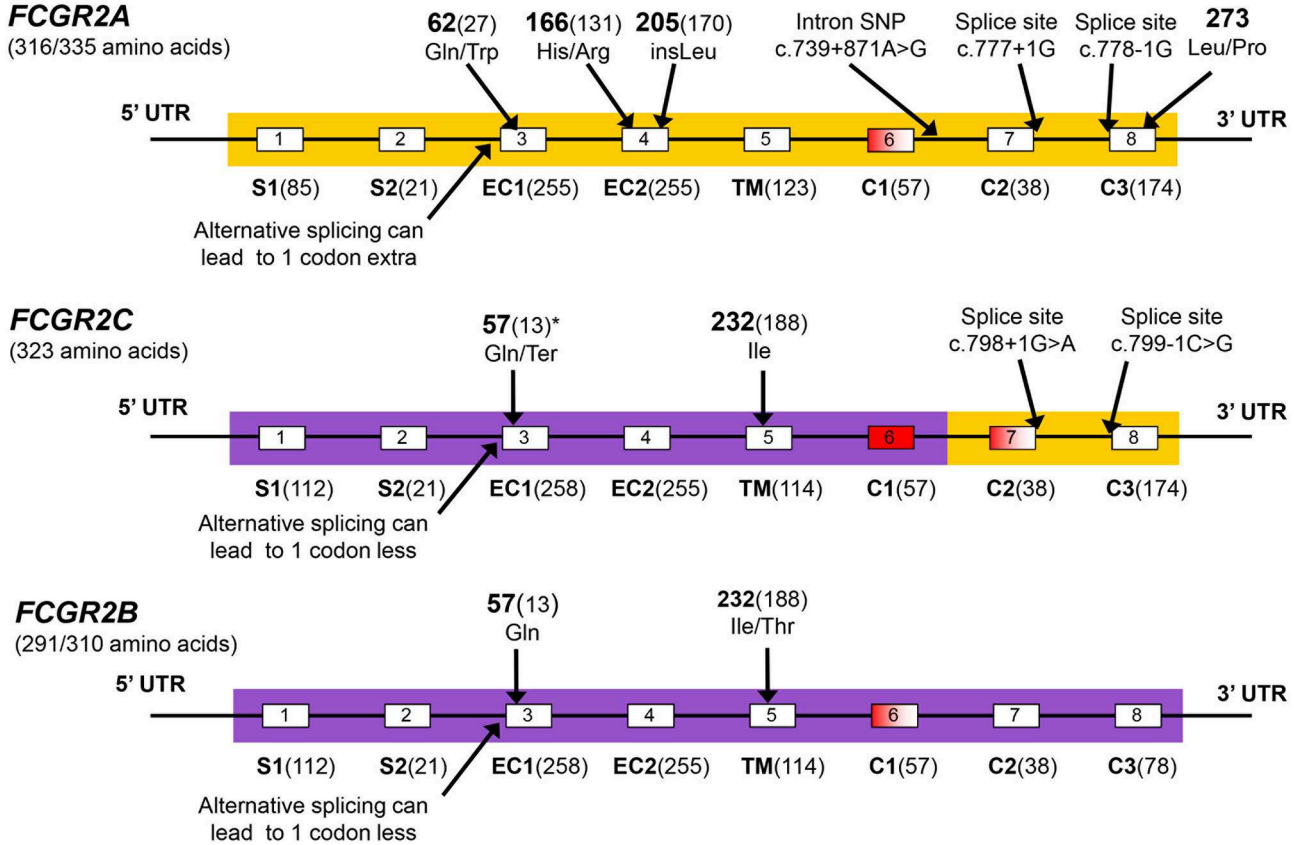

FIGURE 3 | *FCGR2* exons. The *FCGR2C* gene is the crossover product from an unequal crossover between *FCGR2A* and *FCGR2B*. Coloring of *FCGR2C* matches the color of the other *FCGR2* genes in the parts where it is highly homologous to that gene. Exons are shown by boxes, white exons are included in all transcripts, red exons are always spliced out, red-shaded exons are spliced out in some transcripts but retained in others. Exon names are below and followed by the number of coding base pairs in that exon. S1, S2, signal peptides; EC1, EC2, extracellular domains; TM, transmembrane domain; C1, C2, C3, cytoplasmic domains. The C3 exons contain an immunoreceptor tyrosine-based activation motif (ITAM) in *FCGR2A* and *FCGR2C*, and contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in *FCGR2B*. There is a potential confusion with regard to exon numbering in *FCGR2* genes: In the *FCGR2B* gene, transcripts exist that do (FCGR2B1) or do not (FCGR2B2) retain the 57 bp exon6, dependent on the cell type in which the receptor is expressed. In *FCGR2A* and *FCGR2C* a very homologous exon6 is present on genomic level, but this exon is always spliced out in FCGR2A and FCGR2C transcripts. Only in some rare cases exon6 is retained in FCGR2A, which results in a gain-of-function FcγRIIA isoform (39). The final two exons of *FCGR2A* and *-2C* are often designated exon6 and 7, but to reflect the homology between the 3 *FCGR2* genes, we chose to include the potential exon6 in the nomenclature of all *FCGR2* genes, designating the final 2 exons exon7 and 8. However, we do not include the base pairs of this exon6 when indicating the nucleotide positions in the FCGR2A and FCGR2C transcripts, because this is not normally done in the literature. In FCGR2A transcripts, further inconsistencies exist as a result of alternative splicing at the beginning of exon3 because of two adjacent splice acceptor sites that can both be used. The most commonly used nucleotide and amino acid numbering is derived from the shorter transcript in which the 3' splice acceptor site is used, so we chose to use this transcript for nucleotide numbering throughout this thesis. Several SNPs are indicated in the figure, all are indicated by their amino-acid position in the full protein, followed by the amino acid position excluding signal peptides between brackets for some of the SNP which are commonly known by that position. *The p.Gln57Ter SNP in *FCGR2C* is part of a haplotype of 8 SNPs in intron2 and exon3 in *FCGR2C*, this whole haplotype is identical to *FCGR2B* in the case of p.57Gln (40). p57Gln is the only non-synonymous coding SNP in this haplotype.

Nomenclature of Variations at the *FCGR2/3* Locus

Many inconsistencies in the nomenclature of SNPs at the *FCGR2/3* locus exist, because some SNPs are commonly indicated by the amino acid position in the mature protein (from which the signal peptides have been cleaved off), whereas others are indicated by the amino acid position in the full protein. For some SNPs, both positions are used in the literature, which leads to confusion. We propose to use the amino acid positions in the full protein to avoid possible misunderstanding, following the guidelines of the Human Genome Variation Society (HGVS) nomenclature (100) and have used these positions throughout this review. **Table 1** lists also the frequently used positions in the mature protein for some of the SNPs. Further confusion can derive from alternative exon numbering in *FCGR2* genes, as explained in **Figure 3**.

Genetic Analysis of the *FCGR2/3* Locus

As a result of the high sequence homology between the genes, genotyping of this locus is very complicated, and it is important to realize that commonly used genome databases such as Ensembl or NCBI BLAST are not in all cases accurate in the distinction

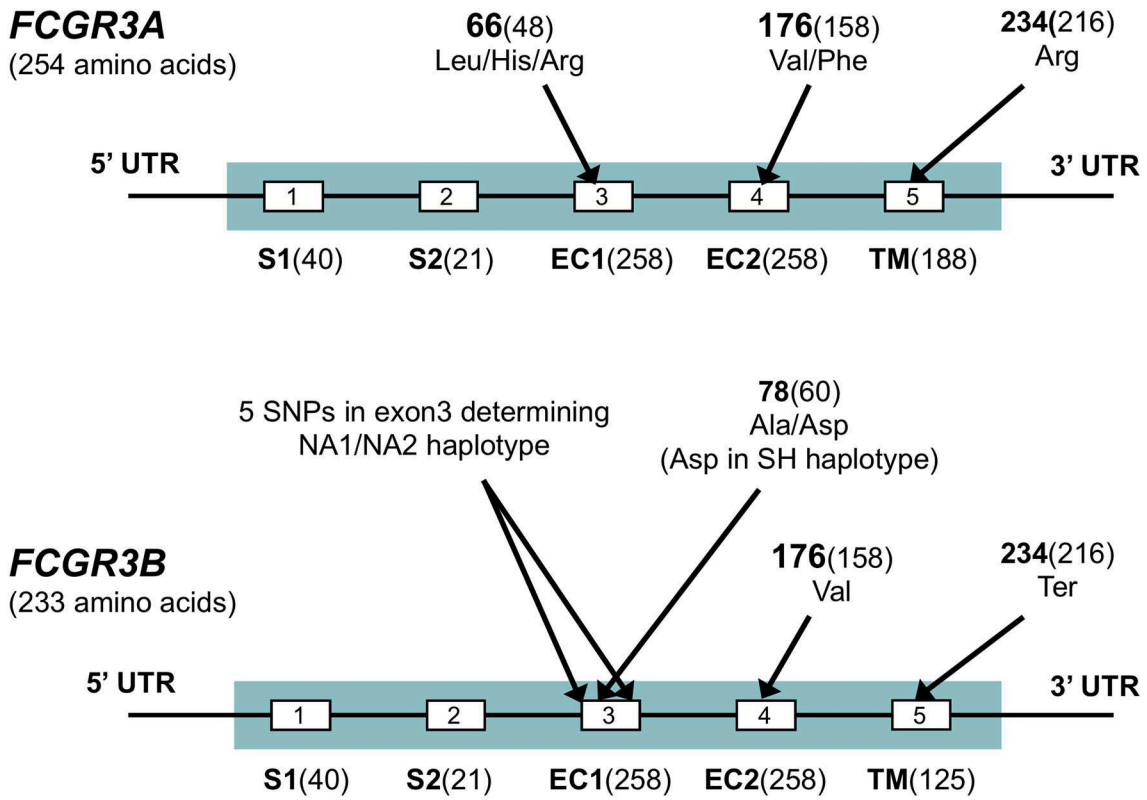

FIGURE 4 | *FCGR3* exons. Exons are shown by boxes. Exon names are below and followed by the number of coding base pairs in that exon. S1, S2, signal peptides; EC1, EC2, extracellular domains; TM: transmembrane domain. Several SNPs are indicated in the figure, all are indicated by their amino-acid position in the full protein, followed by the amino acid position excluding signal peptides between brackets for some of the SNP which are commonly known by that position. Sequences for *FCGR3A* and *FCGR3B* are very similar, but four non-synonymous differences in the coding sequence exist, most notably the stop codon at p.234 in *FCGR3B* as indicated in the figure, which truncates the transmembrane domain of this receptor. Other amino acid differences between *FCGR3A* and *FCGR3B* include p.147 (Gly in *FCGR3A*, Asp in *FCGR3B*), p.158 (Tyr in *FCGR3A*, His in *FCGR3B*) and p.203 (Phe in *FCGR3A*, Ser in *FCGR3B*) (41). A set of 6 SNPs in exon3 of *FCGR3B* form three rather well-defined haplotypes, the *FCGR3B*-NA1, -NA2 and -SH. *FCGR3A* is identical to NA1 at some sites, but to NA2 at others (41).

between a SNP in one of the *FCGR* genes and a genuine difference between two homologous *FCGR* genes (PSV). Detailed knowledge of the organization of the locus and adequate primer design that enables distinction between the paralogs is essential for a proper genetic analysis, and recommendations for analyzing this complex locus have been published before (101). A good source for distinguishing SNPs from PSVs is provided in a Supplementary Table in the article by Mueller et al. (99).

Copy number variation at the locus is commonly determined by multiplex ligation-dependent probe amplification (MLPA) or by paralogue ratio test (PRT) which were concordant in most but not all cases (102). Recently, a method was described that uses data from whole-genome array comparative genomic hybridization (aCGH) to distinguish heterozygous deletion alleles that either include *FCGR3A* (i.e., CNR2 and CNR3) or include *FCGR3B* (i.e., CNR1 and CNR4) (81), which allows for a more high throughput method to determine CNV at the *FCGR2/3* locus, although only heterozygous deletion alleles could be called with reasonable accuracy. An attempt has also been made to determine *FCGR3A* and *FCGR3B* CNV from intensity values derived from an Immunochip platform, allowing the authors to determine CNV in >18,000 individuals (103). This was said to reliably identify cases with 0,1,2 or more copies, although 3 copies could not be reliably be distinguished from higher copy numbers. The authors did however not validate their findings with standard techniques to determine CNV at the *FCGR2/3* locus, such as MLPA or the paralogue ratio test (PRT) (104). In our opinion, the fact that in this study the authors could not find any relation of *FCGR3B* CNV with expression of FcγRIIIB on neutrophils puts serious doubts on the reliability of these data, as expression of FcγRIIIB clearly correlates with CNV of *FCGR3B* as measured by PRT (105) and MLPA (11, 43, 86).

Similarly, next generation sequencing techniques are currently insufficient to determine *FCGCR2/3* SNPs, as multiple variants were mistyped in subjects that were genotyped by whole-exome sequencing (56). Thus, it appears that next generation sequencing techniques will have to be improved greatly before such methods can be used to adequately genotype the *FCGR2/3* locus (as well as other complicated loci with duplications and high homology),

TABLE 1 | Overview of single nucleotide polymorphisms (SNPs) and copy number variation (CNV) at the *FCGR2/3* locus.

Rs #	Nucleotide*	Amino acid position**	Amino acid	Functional change	Associations with disease
FCGR2A					
rs201218628	c.184C c.185A	**62** (27)	Gln	Possibly reduced signaling (42), no influence on expression (43)	
	c.184T c.185G		Trp		
rs1801274	c.497A	**166** (131)	His	higher affinity for human IgG (44)	**KD (45)**, **childhood ITP (46, 47)**, possibly Guillain Barre Syndrome (48, 49)
	c.497G		Arg		**SLE (50, 51)**, meningococcal sepsis (52), sepsis (53, 54)
rs150311303	c.612_613 insCTT	**205** (170)	Leu	higher affinity for human IgG (55)	
rs72717038	c.739 +871 A>G	–	–	G retains exon 6, increased signaling (39)	Anaphylaxis in patients with hypogammaglobulinemia (39)
rs382627	c.818C	**273**	Leu		
	c.818T		Pro	Introduced by deletion of CNR2, decreased expression (56)	
FCGR2B					
rs143796418	−386 C>G***	–	–	Promoter haplotypes 2B.1, 2B.2 and 2B.4 influences expression (57, 58)	2B.4 haplotype associated with susceptibility to SLE (57, 58)
rs780467580	−120 T>A***	–	–		
rs755222686		**106**	Asn		
	c.316_318del		Del	Deletion abolishes IgG-binding	Increased serum levels of IgG1 and IgG3 (59)
rs1050501	c.695T	**232**	Ile		
	c.695C		Thr	Excludes receptor from lipid rafts (60, 61)	**Susceptibility to SLE (50, 60)**, protection against malaria (62)
FCGR2C					
CNV	–	–	–	Expression levels (only in *FCGR2C*-ORF) (63)	KD (unexplained mechanism) (64)
rs149754834	−386 C>G***	–	–	Promoter haplotypes 2B.1, 2B.2 (possibly 2B.4) functional change unknown	
rs34701572	−120 T>A***	–	–		
rs759550223	c.169T	**57** (13)	Ter	Stop codon, no expression of FcγRIIC	
	c.169C		Gln	Results in an open reading frame (ORF) and expression of FcγRIIC (9, 19)	ITP (19, 65), Kawasaki Disease (43), IgG subclass deficiency (66)
rs114945036	c.134-96C>T	–		unknown	Minor allele associated with HIV disease progression (67)
rs138747765	c.353C>T	**118**	Ile/Thr	unknown	Possibly linked to rs114945036
rs76277413	c.798 +1 A>G	–	–	A causes exon7 to be spliced out (9)	
rs430178	c.799−1 C>G	–	–	C leads to retention of 62 intronic base pairs (9)	
FCGR3A					
CNV	–	–	–	Expression levels Decreased ADCC (1 copy vs. 2 copies)	SLE (both <2 and >2 copies) (68), anti-GBM disease (>2 copies) (69)
rs10127939	c.197T	**66** (48)	Leu	Linked to rs396991 (70)	
	c.197A		Arg	Increased IgG binding in presence of *FCGR3A*-p.176Val (71)	
	c.197G		His	Increased IgG binding in presence of *FCGR3A*-p.176Val (71) May result in functional defects in NK cells (72, 73)	Homozygosity associated with severe Herpes infections (72–74)
rs396991	c.526G	**176** (158)	Val	higher affinity for human IgG (44, 70)	Susceptibility to ITP (19, 47, 75), RA (76) and ulcerative colitis (77)
	c.526T		Phe		**Susceptibility to SLE (50)**

(Continued)

TABLE 1 | Continued

Rs #	Nucleotide*	Amino acid position**	Amino acid	Functional change	Associations with disease
FCGR3B					
CNV	–	–	–	Expression levels Uptake of immune complexes	**SLE (<2 copies) (78), Sjögren syndrome (<2 copies) (79), systemic sclerosis (<2 copies) (80)**, **RA (<2 copies) (78, 81, 82)**, Ulcerative Colitis (<2 copies) (83), Ankylosing Spondylitis (84), ANCA-associated vasculitis (< 2 copies) (85) Bullous Pemphigoid (inverse relation with CNV) (86)
rs200688856	c.108G	**36**	Arg	NA1****	
	c.108C		Ser	NA2 and SH****	**NA2 associated with susceptibility to SLE (50)**
rs527909462	c.114C	**38**	Leu	NA1	
	c.114T		Leu	NA2 and SH	
rs448740	c.194A	**65**	Asn	NA1	
	c.194G		Ser	NA2 and SH	
rs5030738	c.233C	**78**	Ala	NA1 and NA2	
	c.233A		Asp	SH	
rs147574249	c.244G	**82**	Asp	NA1	
	c.244A		Asn	NA2 and SH	
rs2290834	c.316G	**106**	Val	NA1	
	c.316A		Ile	NA2 and SH	

Associations found in meta-analyses or GWAS studies are indicated in bold.

For each SNP, Rs numbers (Reference SNP cluster ID, the common identification method of SNPs as included in the dbSNP database), nucleotide and amino acid positions, functional changes, and associations with disease are shown.

**Nucleotide numbering excludes exon6 in FCGR2A and FCGR2C transcripts, because this exon is spliced out from these transcripts, but includes exon 6 in FCGR2B, in which it is retained in many transcripts (splice variant known as FCGR2B1).*

In FCGR2A transcripts, inconsistencies exist as a result of alternative splicing at the beginning of exon3 because of two adjacent splice acceptor sites that can both be used. The most commonly used amino-acid numbering is derived from the shorter transcript in which the 3′ splice acceptor site is used, so we chose to use this transcript for nucleotide numbering throughout this manuscript.

***Inconsistencies exist in the amino-acid numbering used in the literature, because some SNPs are named by the position when including the signal peptides, and others are named by their position in the mature protein, excluding the signal peptides. To comply with the official HGVS guidelines, we propose to use the amino acid in the full protein and have done this throughout the manuscript. In this table, position in the mature protein is shown between brackets for some of the SNP which are commonly known by that position.*

****Relative to the start of translation. Three haplotypes have been described: 2B.1 (−386, −120T); 2B.2 (−386C, −120T) and 2B.4 (−386C, −120A). −386G, −120A has never been found to date.*

*****The set of 6 SNPs in FCGR3B determines the NA1,NA2 and SH haplotypes. These are the three major haplotypes that exist, although rare additional variants have been reported (87). The term "NA" is derived from "Neutrophil Antigen." The term "SH" derives from the fact that an alloantibody recognizing this antigen was first found in serum "h" among several different investigated sera (Jürgen Bux, personal communication). FCGR3B-NA1 and -NA2 nucleotide sequences differ at five positions (c.108G>C, c.114C>T, c.194A>G, c.244G>A and c.316G>A), with four predicted amino acid differences (p.Arg36Ser, p.Asn65Ser, p.Asp82Asn and p.Val106Ile for NA1 and NA2, respectively). As a consequence, the NA2 variant has two additional N-linked glycosylation sites compared to NA1 (the p.65Ser of NA2 completes a consensus sequence for N-linked glycosylation with the non-polymorphic p.63Asn residue, and the p.82Asn of NA2 forms a consensus sequence with the non-polymorphic p.84Ser) (88). The SH variant is identical to NA2 at the five positions that distinguish NA1 from NA2, but differs from both variants at one additional position (c.233C>A), resulting in an p.Ala78Asp amino acid change that predicts a change in the tertiary structure of the protein (89). Additional complexity is added by the discovery of rare individuals carrying other mutations within this gene or different combinations of these nucleotide polymorphisms (87, 90), indicating that the NA1/NA2/SH typing is incomplete. Sometimes, the NA1/NA2/SH haplotypes are indicated, respectively, as FCGR3B*01, FCGR3B*02 and FCGR3B*03, to prevent confusion with the nomenclature for antigenic epitopes determined by these haplotypes. These haplotypes determine the allotypic variants of the Human Neutrophil Antigen1 (HNA1), which is involved in allo-immunization against neutrophilic granulocytes. The HNA classification system recognizes HNA1a (encoded by FCGR3B-NA1), HNA1b (encoded by FCGR3B-NA2 and FCGR3B-SH) and HNA1c (encoded by FCGR3B-SH) (41, 89, 91–93). Recently, a fourth antigenic epitope was described (HNA1d, also encoded by FCGR3B-NA2) (93).*

Note that the nucleotide positions as indicated here are indicating the position in the coding sequence, which differs from nucleotide positions often used in the literature for these haplotypes, as derived from Ravetch and Perussia (94) who used a nucleotide numbering not related to the coding sequence of FCGR3B, which includes 33 additional nucleotides of the 5′UTR.

and it is not sure whether such methods will be present for high-throughput analysis in the near future.

FUNCTIONAL CONSEQUENCES OF SNPS IN THE *FCGR2/3* GENES

In *FCGR2A*, encoding for FcγRIIA, a well-known SNP rs1801724 is present which results in either a histidine or an arginine at position 166 in the full protein: p.His166Arg (formerly known as p.His131Arg). p.His166Arg is in the IgG binding domain (EC2) (106); FcγRIIA-p.166His has a higher binding affinity for IgG1 and especially IgG2, as compared to FcγRIIA-p.166Arg, but binding to IgG3 and IgG4 is similar for both variants (44). Functionally, mononuclear cells from homozygous FcγRIIA-p.166His individuals produce more IL-1beta when stimulated with IgG2 than heterozygous and homozygous FcγRIIA-p.166Arg individuals (107). Similarly, neutrophils from homozygous FcγRIIA-p.166His individuals have been shown

to have increased phagocytosis and degranulation in response to serum-opsonized bacteria and increased rosette formation and phagocytosis in presence of IgG3 anti-RhD sensitized erythrocytes when compared to homozygous FcγRIIA-p.166Arg individuals (108, 109).

In addition to the well-studied *FCGR2A*-p.His166Arg, several other functional SNPs have been described in *FCGR2A*: *FCGR2A*-p.Gln62Trp (formerly known as p.Gln27Trp), a combined SNP of two adjacent nucleotides (known separately as rs9427397 and rs 9427398, and combined as rs201218628) is in linkage disequilibrium with *FCGR2C*-ORF and the *FCGR2B* promoter haplotype 2B.4 (43). Compared to p.62Gln, the p.62Trp allele shows similar FcγRII expression amongst neutrophils and monocytes and, even though slightly reduced calcium signaling has been observed in overexpressed cell lines, does not affect neutrophil ADCC *in vitro* (42). Because of the linkage disequilibrium and given these functional data, it is more likely that 2B.4 variant or *FCGR2C*-ORF confer the increased risk for autoimmune disease than *FCGR2A*-p.62Trp.

At position p.273, which normally is a Leucine in *FCGR2A*, a Proline can be introduced into *FCGR2A* by deletion of CNR2 which causes the fusion of the proximal part of *FCGR2A* and the distal part of *FCGR2C* causing a chimeric *FCGR2A/2C* gene (56). The Leu/Pro difference is the only amino acid different in this region of *FCGR2A* and *FCGR2C*, although additional variants exist in the 3'UTR (56). The chimeric *FCGR2A/2C* gene shows lower expression levels and lower generation of reactive oxygen species in comparison with the wild-type *FCGR2A*. This variant could either be seen as a SNP or as the result of the fusion of two genes. *FCGR2A*-P.Leu273Pro has also been described as a SNP (rs382627, currently flagged as suspect in dbSNP) in a Japanese individual, although it has not been tested whether this individual also had a deletion of CNR2 (110). Compared to leucine, the proline variant was shown in this report to have an increased signaling capacity when expressed in cell lines (110).

Another SNP that may influence the function of FcγRIIA is the intronic rs72717038, which could cause retention of exon6 which is associated with increased signaling capacity (39, 111).

Finally, *FCGR2A*-p.Val204_Gln205insLeu (rs150311303) is a SNP observed with a minor allele frequency of 8.3% in an African population and confers a higher affinity for human IgG (55).

FCGR2B, encoding for FcγRIIB, also exists in two allelic variants, containing either an isoleucine or a threonine at position 232 in the TM domain (112). As this SNP (p.Ile232Thr) does not affect the IgG-binding EC domains, it has no influence on the binding affinity. However, its localization at the TM domain results in differences in downstream signaling and subsequent inhibition of FcγRI signaling in macrophages and BCR signaling in B cells. In particular, p.Ile232 provides stronger inhibitory signaling than p.Thr232, and this is caused by the exclusion from lipid rafts of FcγRIIB-p.Thr232 (60, 61) (**Figure 5**). Dendritic cells (DC) also express FcγRIIB, affecting DC maturation end T cell stimulation (113–115), hence FcγRIIB-p.Thr232 may also influence the function of these cells. Other genetic variations influence the *expression* of FcγRIIB. For instance, in individuals with a CNR1 deletion in the *FCGR* locus, FcγRIIB can surprisingly also be expressed on the surface of NK cells (9, 11, 99). Expression of FcγRIIB in neutrophils,

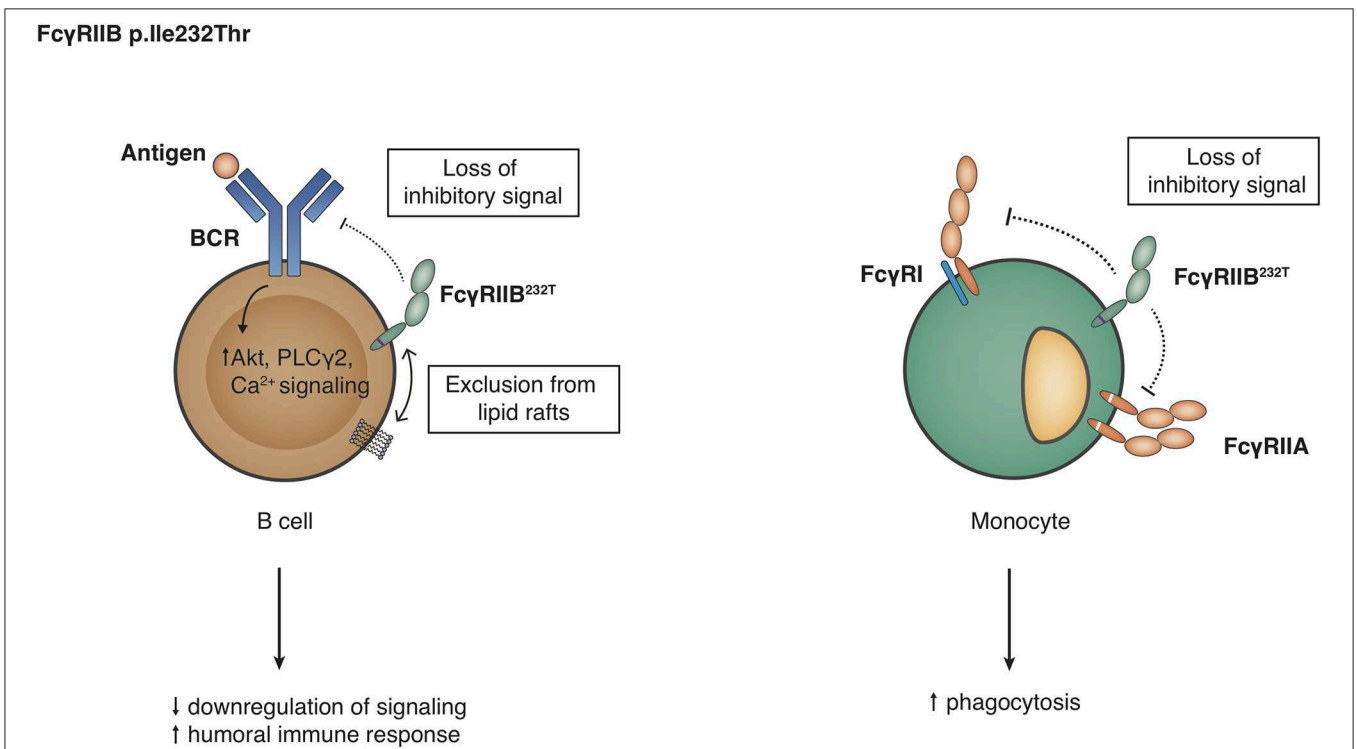

FIGURE 5 | Functional consequences of the *FCGR2B*-p.Ile232Thr variant. Detailed explanations are given in the main text.

monocytes or B cells is hardly affected by this deletion (9). Furthermore, two SNPs in the promoter of *FCGR2B* and *FCGR2C*, a guanine or cytosine at position −386 and a thymine or adenine at position −120 relative to the start codon, form four haplotypes of which one (−386G, −120A; 2B.3) has never been found in any individual thus far. In case of *FCGR2B*, the rare 2B.4 promoter haplotype (−386C, −120A) appeared to have higher transcriptional activity than the wild-type promoter 2B.1 (−386G, −120T) (57), resulting in increased expression on neutrophils (10, 11), monocytes (11) whereas the result of this 2B.4 promoter on the expression of FcγRIIB on B cells is less clear and conclusions from various reports range from increased expression (57), no effect (11) to a *decreased* expression of FcγRIIB on B cells (58); which may differ among different B cell subsets. Functionally, 2B.4 led to a stronger inhibition of B cell receptor signaling without affecting surface expression levels as such (116).

Recently, a rare in-frame deletion c.316_318del, p.Asn106del, rs755222686, was described in the Icelandic population that abolishes IgG binding to FcγRIIb (59). The asparagine residue at position 106 is part of an N-linked glycosylation site, but the absent binding of IgG was not a result of the removal of the glycan, because the same glycan was found at the adjacent asparagine at position 105 in protein encoded by the deletion allele. p.Asn106del was associated with increased levels of IgG1 and IgG3.

In *FCGR2C*, the previously mentioned p.Gln57Ter SNP (sometimes known as p.Gln13Ter determines whether or not individuals can express FcγRIIC at all. This mutation results in either an open reading frame (classic *FCGR2C*-ORF, allele frequency ∼10–15% in Caucasians) or a stop codon (*FCGR2C*-Stop) (19). Classically, ORF/Stop genotyping of individuals is done based on this SNP alone. However, we have recently found that some individuals carry splice site mutations in intron7, which leads to alternative transcripts, causing a frameshift in exon8 and the introduction of novel stop codons, leading to an almost complete loss of FcγRIIC expression (9, 43). Genotyping of *FCGR2C* should therefore include these novel mutations to provide an accurate prediction for FcγRIIC expression.

FcyRIIC on NK cells offers an antibody-mediated, FcyRIIIA-independent pathway to trigger Ca^{2+} signaling and ADCC (16, 117) (**Figure 6**). Because of the identity of the intracellular signaling elements of FcyRIIC and FcyRIIA, it can be speculated that FcyRIIC acts as an independent phagocytic receptor on myeloid cells, but this has not been shown experimentally. Transgenic mice expressing FcyRIIC in B cells show enhanced humoral immune responses after T cell dependent and T-independent vaccination (18). Altogether, classic *FCGR2C*-ORF may thus predispose to autoimmune disease either by providing attenuated innate immune responses or by enhancing the humoral immune response.

In *FCGR2C*, the promoter haplotypes of *FCGR2B* as mentioned above, can also be found. In general, only the wildtype and one other promoter haplotype (−386C, −120T; 2B.2) are found. The 2B.2 haplotype is linked to p.57Gln (19, 43).

Recently, a haplotype of several SNPs in and around exon3 has gained a lot of attention because of a possible association with HIV vaccine efficacy. This haplotype consists of c.134-96C>T (rs114945036), p.(Thr118Ile) (rs138747765) and c.391+111G>A (rs78603008) and was associated with vaccine efficacy despite the fact that all but one study participants had a p.57Ter allele. The function of the SNPs in the haplotype is unknown. However, the results of this study must be taken with great caution because the primers used to distinguish *FCGR2C* from *FCGR2B* have used specific sites that, according to Mueller et al. (99), are all polymorphic in *FCGR2C* and can completely resemble *FCGR2B*. The authors may therefore have missed some *FCGR2C* alleles in their analysis which may have skewed the results. Later, the haplotype has been reanalyzed in a South African cohort (118) using a specific long range PCR to sequence *FCGR2C*; these authors have indeed found the c.134-96C>T SNP but not the other SNPs of the haplotype in their cohort. The minor allele of the c.134-96C>T SNP was later found by the same group to be associated with increased odds of HIV-1 disease progression (67).

The FcγRIIIA-encoding *FCGR3A* gene contains a SNP that results in either a valine or a phenylalanine at position 176 (p.Val176Phe), formerly known as p.Val158Phe, located in the EC2 domain (119). FcγRIIIA-p.176Val has a higher binding affinity for all human IgG classes compared to FcγRIIIA-p.176Phe (44). In ADCC assays, NK cells from FcγRIIIA-p.176Val donors show increased killing of target cells that are opsonized with sub-saturating levels of the human anti-CD20 MoAb Rituximab (23). Another SNP in the *FCGR3A* gene is a triallelic SNP at position 66; p.66Leu/Arg/His, also formerly known as p.48Leu/Arg/His which is located in the EC1 domain which is not directly involved in binding IgG. Rare homozygosity of p.66His was first described in a patient with recurrent Herpes infections (72) and was later found in two other patients with decreased clearance of Herpes infections (73, 74) and suggested to be a congenital immunodeficiency (73). However, homozygosity for p.66His was also found in a cohort of healthy individuals of European descent (genotype frequency 0.6%) and African descent (genotype frequency 0.1%) (71). Apparently, the clinical phenotype of homozygity for p.66His differs between individuals and recurrent Herpes infections may be associated with but are not directly caused by the mutation.

The FcγRIIIB-encoding *FCGR3B* gene exists in three polymorphic variant proteins, best known as the NA1, NA2, and SH haplotypes. These haplotypes consist of a set of 6 SNPs in exon3 of *FCGR3B* (**Table 1** and **Figure 4**). The *FCGR3B* variants encoded by these haplotypes determine the allotypic variants of the Human Neutrophil Antigen1 (HNA1) (91), which is involved in allo-immunization against neutrophilic granulocytes. The NA1, NA2 and SH haplotypes are sometimes referred to in the literature as HNA1a, HNA1b and HNA1c, respectively, although the latter nomenclature in strict sense determines antigenic epitopes and not genetic haplotypes (see **Table 1** for a detailed description). Apart from determining allo-immunization against neutrophils, these haplotypes are known to have functional differences. Compared to NA1, the NA2 and SH variants have two additional N-linked glycosylation sites. The SH variant differs from NA1 and NA2 by a p.Ala78Asp amino acid change

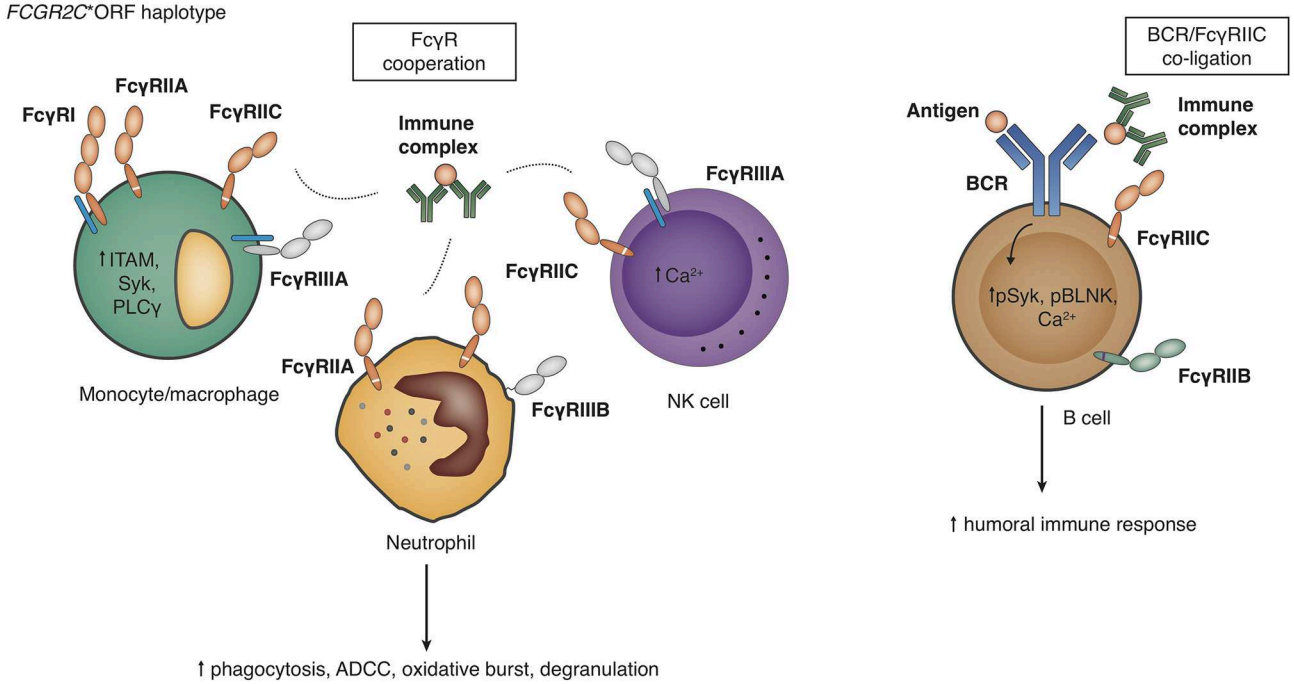

FIGURE 6 | Functional consequences of the *FCGR2C*-open reading frame (ORF) haplotype. Detailed explanations are given in the main text. Right panel, Effects on the level of B cells have been described by Li et al. (18), but other analyses have not shown FcγRIIC expression on B cell surface in *FCGR2C*-ORF individuals (17).

that predicts a change in the tertiary structure of the protein (89), although the actual functional consequences of this SNP are not well-characterized. While the binding affinities for IgG1 and IgG3 appear similar between NA1, NA2 and SH (44), neutrophils from FcγRIIIB-NA1NA1 individuals bind and phagocytize IgG-opsonized bacteria and red blood cells more efficiently than those from FcγRIIIB-NA1NA2 and -NA2NA2 individuals (108, 120). It is not known whether the SH is functionally different from the otherwise similar NA2 variant.

FUNCTIONAL CONSEQUENCES OF CNV IN THE *FCGR2/3* GENES

CNV (**Figure 2**) results in differences in expression levels of FcγRIIIB, where an increase in gene copies of *FCGR3B* very clearly leads to a higher receptor expression at the cell surface (11, 63, 105, 121, 122) and higher mRNA levels (43, 86), and decreased copy number associated with lower soluble serum FcγRIIIB levels released from activated neutrophils (123) (**Figure 7**). Functionally, increased expression of FcγRIIIB leads to higher binding and uptake of immune complexes by neutrophils (105) and may be associated with increased ROS production (86), which is an intriguing finding because FcγRIIIB has been shown not to contribute to ROS production (27). Interestingly, individuals who are homozygous for a CNR1 deletion, i.e., have no copies of *FCGR3B*, are generally healthy and apparently not at risk for overwhelming bacterial infections, suggesting that their neutrophil function is sufficient to maintain immune homeostasis (122).

In addition, a CNR1 deletion leads to expression of FcγRIIB on NK cells (9, 99), which is presumed to result from the molecular effects of the promoter rearrangement, and the ectopic expression of FcγRIIB is either regulated by action of the *FCGR2C* promoter or by deletion of a negative regulatory elements in the *FCGR2B* promoter. Humans with CNR1 deletion show a reduced FcγRIIIA-mediated NK cell ADCC, and FcγRIIB expression on NK cells was able to fully inhibit redirected FcγRIIC-mediated ADCC (9).

In case of FcγRIIC, CNV can only play a role in case of classic *FCGR2C*-ORF alleles, but in these cases the amount of copies of *FCGR2C*-ORF alleles is clearly linked to expression values (19, 43).

In FcγRIIIA, surface expression of FcγRIIIA on NK cells is also linked to gene copies of *FCGR3A* (63), although the expression levels are not as clearly associated as in *FCGR3B* and no significant correlation with mRNA levels could be found (43) suggesting some form of transcriptional regulation. The level of expression on NK cells is, at least for 1 vs. 2 copies, related to the level of killing of target cells in (redirected) ADCC assays (63).

LINKAGE DISEQUILIBRIUM AT THE *FCGR2/3* LOCUS

With all the *FCGR2* and *FCGR3* genes so closely associated in the *FCGR2/3* locus, the different SNPs and CNRs are prone

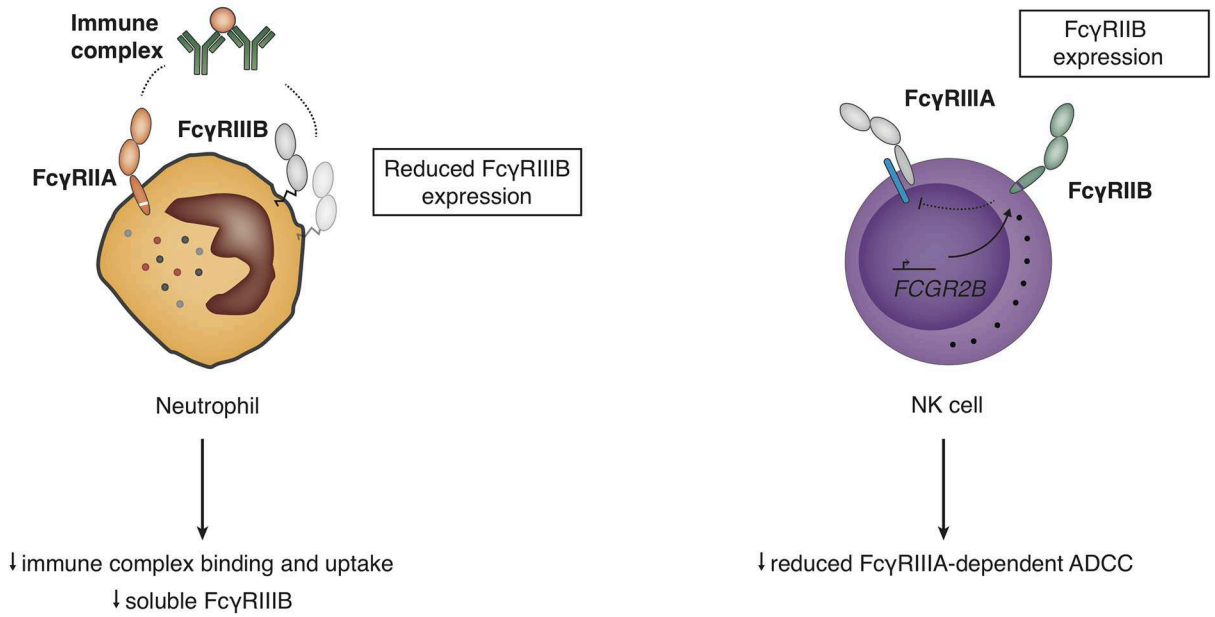

FIGURE 7 | Functional consequences of deletion in the copy number region 1 (CNR1). Detailed explanations are given in the main text.

to have a high degree of linkage disequilibrium (LD), and we have recently published a large overview of LD at the locus (43), which showed LD to occur across the locus. The LD found was similar to LD previously found between some selected sets of variants (98, 124, 125). In addition, the *FCGR3A*-p.66Leu/His/Arg SNP has been shown to be in LD with *FCGR3A*-p.Val176Phe (70), and this linkage was responsible for an initially observed difference in binding affinity for IgG in *FCGR3A*-p.66Leu/His/Arg (126), which was later shown to be solely the result of the linkage with the *FCGR3A*-p.Val176Phe (70), which conferred the difference in binding affinity. However, later binding assays with stratified groups of individuals showed some direct differences in binding affinity resulting from the *FCGR3A*-66Leu/His/Arg itself (71). Thus, knowledge of LD is very important for a correct interpretation of genotyping results.

Consideration of linkage disequilibrium should have a great impact on all the association studies performed for variants at this locus, which often genotype one or two variants only (mostly the *FCGR2A*-p.His166Arg and *FCGR3A*-p.Val176Phe SNPs). Associated variants found in these studies may be the mere reflection of an even stronger association for a variant that is in LD with the identified variant, but was not genotyped in the study (i.e., an increased prevalence of *FCGR3A*-p.176Val in a certain patient group may simply be the result of the classic *FCGR2C*-ORF haplotype being even more increased in the same patient group, because these two variants are in LD with each other). If only the *FCGR3A*-p.Val176Phe SNP is genotyped, this could lead to the wrong conclusion that *FCGR3A*-p.176Val causes an increased susceptibility to the disease. To be able to most accurately identify a potentially causative variant for an increased disease susceptibility, all the known functional genetic variants at the *FCGR2/3* locus have to be genotyped, as we have done with the MLPA technique in our studies. A multiple logistic regression analysis can then identify independent risk markers. Even then, for variants that are in strong LD with other variants, it may be hard to identify independently associated variants, and large groups are needed. For instance, in single logistic regression analyses we observed four variants that are in strong LD with each other, to be all associated with an increased susceptibility to Kawasaki Disease (KD) (43) as well as Immune thrombocytopenic purpura (ITP) (65). It seems likely that only one of these variants (the classic *FCGR2C*-ORF haplotype) actually causes the increased risk, but to prove this as an independent marker in a multiple logistic regression analysis, many more patients would need to be included in the association study, which is not easily done for rare diseases. On the other hand, for SNPs that are in less strong LD, multiple logistic regression analysis can still identify independent risk markers also in smaller patient groups.

Considering the strong LD and well-defined structure of CNRs at the locus, it may be better to analyze -and report on- some of the genetic variations at the locus as haplotypes, instead of analyzing single variations. This has already been the standard for the SNPs in *FCGR3B*, which are usually reported as the haplotypes NA1, NA2 and SH. Now, since we know that CNV at the *FCGR2/3* locus always occurs in CNRs, we suggest

that CNV should be analyzed in the form of these CNRs, which basically form haplotypes. For instance, CNV in *FCGR3B* never occurs alone, but is always accompanied by CNV of *FCGR2C* and *HSPA7* (in essence, CNV in *FCGR3B* is in perfect LD with CNV in *FCGR2C* and *HSPA7*). Thus, an independent association of *FCGR3B* CNV with disease is impossible to prove, although in this case seems very likely because both the *FCGR2C* and *HSPA7* genes are pseudogenes in the majority of individuals. However, in case of the most frequent deletion allele of CNR1, the ectopic expression of FcγRIIB on NK cells (9, 99) may also play a role in disease susceptibility, and these effects are impossible to distinguish with genetic association studies.

Similarly, a decreased copy number of *FCGR3A*, which most often occurs as part of the copy number region CNR2, is in these cases always accompanied by the newly described *FCGR2A/2C* chimeric gene (56) (in essence, decreased copy number of *FCGR3A* is in very strong LD with the presence of an *FCGR2A/2C* chimeric gene). Concluding, the presence of CNV at the *FCGR2/3* locus cannot be analyzed separately for single genes, and therefore we think it is better to report the CNV as haplotypes in the form of the different CNRs at the locus.

From a functional point of view, *FCGR2C* variations should also be reported as haplotypes (classic *FCGR2C*-ORF, non-classic *FCGR2C*-ORF and *FCGR2C*-Stop). These consist of two (possibly three) SNPs that together determine expression of FcγRIIC (43). Analyzing the *FCGR2C*-p.Gln57Ter alone would include individuals with the non-expressed non-classic *FCGR2C*-ORF haplotype within the group of the classic *FCGR2C*-ORF haplotype, whereas we show that on a phenotypic and functional level, that the non-classic *FCGR2C*-ORF haplotype is similar to *FCGR2C*-Stop. Therefore, these *FCGR2C* variations should always be genotyped together and reported as haplotypes. *FCGR2C*-p.Gln57Ter should not be genotyped alone.

In view of the LD at the locus, and because the FcγR proteins encoded by the genes are functionally related and could thus act together in pathophysiologic mechanisms, it could even be attempted to report extended haplotypes, including all the different SNPs across the whole locus. However, the LD is not absolute for any of the SNPs, and thus the list of possible haplotypes is very long. Therefore, such an approach seems unpractical and confusing. When analyzing the locus as extended haplotypes, disease associations of a single gene encoding one of the FcγRs will not be obvious anymore, which will obscure valuable information on pathophysiology of the disease that is studied.

ETHNIC VARIATION AT THE *FCGR2/3* LOCUS

Several reports have shown that extensive ethnic variation exists at the *FCGR2/3* locus (43, 97, 98), especially for the *FCGR2C* haplotypes (43, 118), which is relevant for genetic association studies, as it emphasizes the importance of carefully selecting ethnicity-matched control groups.

EVOLUTION OF THE *FCGR2/3* LOCUS

The *FCGR2A* gene, defined as a gene that contains an ITAM within its sequence, appears to be specific to primates (127), and has evolved from its ortholog *fcgr3* in non-primate animals by NAHR and integration of a retroviral element that included the ITAM (127). The *FCGR3(A)* gene in primates is an ortholog of non-primate *fcgr4* (127), in fact, in some primate species this gene is also indicated as *FCGR4*. Finally, *FCGR2B* in primates is an ortholog of *fcgr2b* (127). Taken together, the basic structure of the *FCGR2/3* locus in primates is *FCGR2A-HSPA6-FCGR3A*(in some cases known as *FCGR4*)*-FCGR2B,* with *FCGR2A* being by far the most distinct from non-primate genes in this locus (127).

The *FCGR2C* and *FCGR3B* (and pseudogene *HSPA7*) genes appear to have evolved more recently. They were formed in a segmental duplication of the *FCGR2/3* locus that forms the structure *FCGR2A-HSPA6-FCGR3A-FCGR2C-HSPA7-FCGR3B-FCGR2B* (15).

Several studies have tried to determine the presence of this segmental duplication in other primates, with sometimes discordant results. It appears that within the great apes (Hominidae family), only the members that are closest to humans (the Homininae subfamily) have the segmental duplication, as shown in humans, chimpanzees (*Pan troglodytes*) (97, 127) and gorillas (*Gorilla gorilla*) (127), although one study suggested that even chimpanzees did not have the *FCGR2C* and *FCGR3B* genes (128). The other members of the Hominidae family, the orangutans, do not appear to have duplicated the locus (97, 127, 128). A little more distant, the gibbons may however have separate *FCGR3A* and *FCGR3B* genes (97) although this could not be replicated in the lar gibbon (*Hylobates lar*) with gene-specific primers for *FCGR2C* in a recent study (127). Regarding non-hominoid primates, evidence from all studies suggests that the *FCGR3B* gene is not present in macaque species (97, 127–130), nor in baboons (97, 130), with the rhesus macaque (*Macaca mulatta*) (97, 128–130), crab-eating macaque (*Macaca fascicularis*) (97, 127, 129) and hamadryas baboon (*Papio hamadryas*) (130) studied in detail. Confirming these genetic findings, no CD16 expression could be detected on neutrophils from macaques or baboons (130). On the other hand, neutrophils of sooty mangabey (*Cercocebus atys*) did express CD16 (130), which may suggest the presence of an *FCGR3B* gene, although direct genetic evidence for divergence of the *FCGR3* gene could not be found for mangabey (unknown species) (97). With all these studies, it must be taken into account that the complexity of the *FCGR2/3* locus may have precluded authors from finding *FCGR2C* and *FCGR3B* genes in genomes that are not so well characterized.

In any case, the segmental duplication that created the *FCGR2C* and *FCGR3B* genes appears to have occurred relatively recently in evolution and the most thorough and recent study by Lejeune et al. (127) restricts the segmental duplication to Homininae and estimates the event to have occurred <9.2 million years ago. Therefore, it is not so surprising that null variants of these genes are compatible with life in humans; *FCGR2C* is a pseudogene in >80% of the population and healthy human individuals lacking the *FCGR3B* gene have been

described (122). Indeed, a complete lack of the *FCGR3B* gene was found in about 0.2% of individuals in a large cohort of >4,000 individuals (56). However, *FCGR3B* clearly has an important role in the immune system, given the fact that low copy number increases the risk of developing SLE. Apparently, the emergence of *FCGR3B* was beneficial to the species, and this resulted in the fact that the segmental duplication was maintained, and the *FCGR3B* gene has evolved to be very different from the *FCGR3A* gene in expression pattern and function. Evolutionary pressure from helminth infection may have driven this evolution, as an association between large helminth burden and variant frequency in the *FCGR3B* gene was found in human populations (97).

On the other hand, *FCGR2C* seems to be not so beneficial, and this result of the segmental duplication has since been modified by evolution to reduce its function. We assume that with the formation of *FCGR2C* by the segmental duplication of the locus (15), this gene must have been created as a bona fide receptor with an open reading frame. Subsequently, evolution seems to have selected variants that cause reduced function in this receptor through multiple ways: i.e., as a stop codon in exon3, a splice variant in intron7 that abrogates expression (9), and a 3′UTR that does not favor expression when compared with the similar 3′UTR of *FCGR2A* (56). All these changes indicate that having an active FcγRIIC has been selected against, and the classic *FCGR2C*-ORF haplotype may be the last functional remnant of the original *FCGR2C* formed in the segmental duplication. No clear benefits for having an active FcγRIIC are known except for a possible protective effect in helminth infections (97), but it does certainly predispose for certain autoimmune diseases (19, 43). The fact that it does occur at higher frequencies in the European population is intriguing, since it is extremely rare to absent in African populations (43, 118), which constitute the ancestors of the human race. One possibility is that the few classic *FCGR2C*-ORF alleles that may have been present in the European population after the migration out of Africa, were positively selected (or at least selection *against* this variant was less strong) and now represent the increased prevalence when compared to African populations. Other possibilities could be a Neanderthal origin of the classic *FCGR2C*-ORF allele or that it has been newly created in the European population by subsequent recombinations of CNR1 and CNR4, a theory which is supported by the fact that the classic *FCGR2C*-ORF alleles are actually a haplotype of multiple SNPs in intron2 and exon3 that completely resemble *FCGR2B* (40). Considering this possibility, one could predict that *FCGR2B*-Stop alleles (56) were also formed in this way, but the rarity of *FCGR2B*-Stop alleles suggests great evolutionary pressure on this variant, which may be associated with severe autoimmunity.

GENETIC VARIATION IN *FCGR2/3* GENES: ASSOCIATIONS WITH DISEASE

Both SNPs and CNV in *FCGR* genes have been associated with susceptibility to several auto-immune and infectious diseases. Importantly, despite the extensive linkage disequilibrium at the *FCGR2/3* locus, most studies have assessed polymorphisms in relative isolation without a broader consideration of connected variants at the locus. **Table 1** provides an overview for a selection of these associations, concentrating on meta-analyses and novel associations that have not been reviewed before by Gillis et al. (3) and Bournazos et al. (131). In general, most of the studies focus on only one or two SNPs; the *FCGR2A*-p.His166Arg and *FCGR3A*-p.Val176Phe are the most studied.

FCGR2A-p.His166Arg

Individuals with the variant *FCGR2A*-p.166Arg have increased susceptibility to SLE, compared to p.166His (50, 132–134), an association which remained also in a GWAS study (135). Although several studies suggest an increased risk of p.166Arg with the development of lupus nephritis (134, 136–138), conflicting evidence exists and a previous meta-analysis did not confirm an association (132).

In contrast to the association of p.166Arg variant with SLE, *FCGR2A*-p.166His is associated with development of ulcerative colitis (77, 139). Furthermore, a genome-wide association study (GWAS) has revealed *FCGR2A*-p.166His to be strongly associated with susceptibility to Kawasaki disease, a pediatric vasculitis affecting the coronary vasculature in particular (140). This association was confirmed in a GWAS (45). Finally, concerning immune thrombocytopenia, a meta-analysis ascertained an association between *FCGR2A*-p.166His and susceptibility to childhood ITP, but not adult ITP (46, 75, 141, 142). Taking the genetic associations into account, it may be speculated that a reduced function of FcγRIIA with the p.166Arg variant is associated with a failure to clear circulating immune complexes, which is a hallmark of SLE. On the other end, a relatively more active immune response, conferred by *FCGR2A*-p.166His, is associated with the development of ITP, Kawasaki disease and inflammatory bowel disease, emphasizing the intricate role of *FCGR2A* at the interface of multiple pathways leading to autoimmunity.

FCGR3A-p.Val176Phe

A diallelic SNP is responsible for p.Val176Phe polymorphism in *FCGR3A*. For SLE, a recent meta-analysis established a link with disease susceptibility and p.176Phe (50), and the p.176Phe/Phe genotype is associated with development of lupus nephritis (143). In rheumatoid arthritis, *FCGR3A*-p.176Val/Val is associated with susceptibility to the disease amongst Europeans, but not Asians (76). Considering ulcerative colitis, there is a small increased susceptibility to develop the disease with the p.176Val allele (77). Finally, several studies have associated the *FCGR3A*-p.176Val/Val genotype with increased susceptibility for ITP (19, 75, 141, 142).

FCGR2B-p.Ile232Thr

FcγRIIB holds a diallelic SNP that determines the p.Ile232Thr amino acid. The p.232Thr/Thr genotype has been strongly associated with SLE in numerous studies (50, 60, 62, 98, 144–146). Interestingly, the p.232Thr allele is in linkage disequilibrium with deletion of CNR1, but when assessed together both variations are independently associated with susceptibility to SLE (98).

In RA, *FCGR2B*-p.232Thr is not associated with disease susceptibility, but strongly associated with joint damage (147).

Although studies indicated that the p.232Thr allele is increased in adult ITP (148) and in chronic childhood ITP (149), a recent meta-analysis questioned this association (6 studies) (142). However, in a recent study three childhood ITP patients that were treated with IVIg and had the p.232Thr/Thr genotype failed to respond to IVIg, whereas 17 patients who carried the p.232Ile/Ile genotype and were only observed, all had a remarkable complete recovery from ITP during follow-up without any treatment (150). Such spontaneous recovery during observation at that timepoint is observed in ∼20% of individuals overall. These data collectively indicate that the *FCGR2B*-p.Ile232Thr genotype may be associated with prognosis in childhood ITP.

Classic *FCGR2C*-ORF and *FCGR2B* Promoter Haplotype 2B.4

The classic *FCGR2C*-ORF is in strong linkage disequilibrium with the *FCGR2B* promoter polymorphism 2B.4 and a third allele, *FCGR2A*-p.62Trp (43). Only few studies investigated all these variants in the included individuals, and identified relationships between variants can therefore not be distinguished between them.

In adult autoimmune diseases, there is an association of classic *FCGR2C*-ORF with ITP (19). In rheumatoid arthritis, CD32 expression on NK cells correlated with mild disease, as compared to aggressive disease (151). However, besides classic *FCGR2C*-ORF that induces FcyRIIC expression, FcyRIIB expression on NK cells from CNR1 deletions must have contributed to this picture, as not all patients with CD32 expression had an ORF allele. Unfortunately, this was not assessed. Presence of classic *FCGR2C*-ORF was also associated with susceptibility to SLE in one study (18) although this was not found in another study (11). Similarly to RA, the *FCGR2B* promoter polymorphism 2B.4 also correlated with susceptibility to SLE, and patients with this variant showed reduced autoantibody development and development of lupus nephritis (11, 57, 58). Finally, *FCGR2C* has been identified as a candidate susceptibility gene for systemic sclerosis (152).

Regarding childhood autoimmune diseases, classic *FCGR2C*-ORF confers susceptibility to childhood ITP (19, 65) as well as Kawasaki disease (43). Classic *FCGR2C*-ORF and 2B.4 correlate positively to immunomodulatory treatment with response to intravenous immunoglobulins (IVIg) in childhood ITP (65) and, for 2B.4, in Kawasaki disease (153). Moreover, in ITP, the variants are associated with a transient disease course, and negatively associate with chronic thrombocytopenia (65).

When these observations are combined, the linked variants classic *FCGR2C*-ORF and 2B.4 are associated with susceptibility to multiple autoimmune diseases. However, where assessed, they conferred a relatively mild disease phenotype and beneficial association with treatment response to IVIg. This suggests that patients without these variants have other contributing determinants to autoimmunity that confer a relatively negative impact on disease severity.

Deletions in Copy Number Region 1 (CNR1)

Copy number variation in *FCGR3B* can arise from insertions or deletions of CNRs in the *FCGR2/3* locus, namely CNR1 and CNR4. Deletions in CNR4 are extremely rare at ∼ 0.1% of the population, whereas a deletion in CNR1 is present in 8.6% of the population (56). Almost all studies investigating CNR1 have only determined *FCGR3B* CNV, but since virtually all deletions or duplications of *FCGR3B* found by these studies will result from CNV of CNR1 we have used the terms interchangeably in the next paragraphs. As said above, whether the effect driving the association reflects expression levels of FcγRIIIB or the ectopic expression of FcγRIIB on NK cells cannot be determined.

Associations between deletions of *FCGR3B* and susceptibility to adult autoimmune diseases have been found for SLE (78, 85, 98, 105, 154), ulcerative colitis (83), rheumatoid arthritis (RA) (81, 82, 155–157), ankylosing spondylitis (84), systemic sclerosis (80), primary Sjögren syndrome (SS) (79, 155), microscopic polyangiitis and Wegener's granulomatosis (85). In SLE, deletion of CNR1 is associated with a higher frequency of lupus nephritis (158). We also recently established that CNR1 deletion is associated with chronic and IVIg-resistant immune thrombocytopenia, but not with the transient form of the disease (65). Interestingly, also duplications of the *FCGR3B* gene were found to be associated with SLE and SS (155) as well as antineutrophil cytoplasmic antibody-associated systemic vasculitis (105), although this was not evident—or conflicted—by other studies (85, 154). In RA, there seemed to be an association with more rheumatoid factor (RF)-positive disease (157), which was not picked up in a smaller study (82). These effects may be modified by other susceptibility genes such as *CCL3L1* (155) or deletions, such as *ADAM3A* (154). In contrast to these systemic autoimmune disease, no association with a deletion of *FCGR3B* has been found for Graves' disease or Addison's disease (85), which suggests that these organ-specific autoimmune diseases are not influenced by *FCGR3B* copy numbers, although one study found an association of increased copies of *FCGR3B* with the a protection against the skin blistering disease Bullous Pemphigoid (86).

Recent meta-analyses confirmed the association between *FCGR3B* copy numbers and susceptibility with autoimmune diseases for low *FCGR3B* copies for SLE, Sjogren's syndrome and Wegener's granulomatosis (78, 159). These association were similar amongst Caucasians and Asians (159). For RA, evidence may be less clear (160), as previous meta-analyses had disparate results (82, 156), although the most recent meta-analysis from 2012 (82) did find an association and furthermore a recent study describing a large cohort confirmed the association (81).

Overall, deletions of *FCGR3B* as part of CNR1 are strongly associated with development of autoimmune diseases. The notion that this association is particularly pronounced for systemic, but not organ-specific autoimmune reactions, are suggestive of divergent pathomechanisms that may be

TABLE 2 | Effects of genetic variants at the *FCGR2/3* locus on immune function.

	Effect on	More immune activation	Less immune activation
FCGR2A-p.His166Arg	Affinity for IgG	His	Arg
FCGR3A CNV	Expression of activating receptor	CNV > 2	CNV < 2
FCGR3A-p.Val176Phe	Affinity for IgG	Val	Phe
FCGR2C haplotype	Expression of activating receptor	Classic ORF	Stop/ Non-classic ORF
FCGR2B-p.Ile232Thr	Strength of inhibitory signal	Thr	Ile
FCGR2B promotor haplotype	Expression of inhibitory receptor	2B.1	2B.4
FCGR3B CNV	Expression of receptor	?	?
FCGR3B haplotype	Phagocytosis (unknown mechanism)	NA1	NA2

?, no clear net result on immune activation.

triggered by CNR1 deletions and subsequently predispose to autoimmunity.

THE USE OF GENETIC ASSOCIATION STUDIES AT THE *FCGR2/3* LOCUS

FCGR2/3 polymorphisms are useful when investigating the role of FcγRs in human disease by means of genetic association studies. In general, such studies can suggest that FcγRs are involved in the pathophysiology of a certain disease. More specifically, association with specific FcγR genetic variations can give a more precise clue, as they may incriminate a certain cell type in the pathophysiology. The best example is the association of FcγRIIIB CNV with SLE (11, 34, 105, 161). Since FcγRIIIB is expressed almost exclusively in neutrophils, this is a strong suggestion that neutrophils are involved in the pathophysiology of SLE. All the other FcγRs are expressed on multiple cell types, and thus, associations of genetic variation in other genes than *FCGR3B* are less indicative of a specific cellular involvement in a disease. In some cases, it may be only possible to determine whether, in very general terms, the *more activating* or the *less activating FCGR* variants are associated with the disease studied, and thus gain insight on the general role of FcγRs in the pathophysiology of the disease. An overview of the more and less activating variants at the *FCGR2/3* locus is given in **Table 2**. Interestingly, several variants that are more activating are in LD with each other: *FCGR2A*-p.166His, *FCGR3A*-p.176Val, and the classic *FCGR2C*-ORF, although this may be 'balanced' by the LD of the classic *FCGR2C*-ORF with the 2B.4 promoter haplotype in *FCGR2B* (43).

Traditionally, it has been thought that activating and inhibitory FcγRs constitute an immunological balance that ensures adequate protection against pathogens, but on the other hand does not result in auto-immunity (7). Simply speaking, *FCGR2/3* genetic variation may tip this balance to either side, leading to auto-immunity when the balance is tipped toward the activating side, or leading to decreased immunity against pathogens or cancer cells when the balance is tipped toward the inhibitory side. However, this may be an over-simplification of the matter, and marked differences in *FCGR2/3* genetic variations occur between several autoimmune and autoinflammatory diseases. In KD and in immune thrombocytopenia (ITP), it is indeed the case that the more activating variants (*FCGR2C*-ORF, *FCGR3A*-p.176Val, *FCGR2A*-p.166His) are associated with disease susceptibility. However, in other autoinflammatory diseases, the less activating variants, which would be expected to tip the balance toward the inhibitory side, actually predispose to disease. This is for instance the case in SLE, which is associated with the less activating variants *FCGR2A*-p.166Arg, and the *FCGR2B* promoter haplotype 2B.4, which causes increased expression of the inhibitory FcγRIIB (10, 11). Another difference between SLE on the one hand and KD and ITP on the other hand is that SLE is associated with low copy number of *FCGR3B* (as is Sjögren syndrome, systemic sclerosis and possibly RA). Low copy number of *FCGR3B* is not associated with KD or ITP.

Clearly, autoimmunity is not necessarily associated with more activating *FCGR2/3* genetic variations, and *FCGR2/3* variants have different, sometimes opposite, effects on different autoimmune and inflammatory diseases, suggesting different pathophysiologic contributions of IgG and FcγRs between the diseases. Possibly, activating FcγRs actually protect against SLE by enabling "waste disposal" of pathogenic immune complexes involved in the disease. On the other hand, in the diseases in which activating variants are associated (KD and ITP), damage done by IgG may be exerted directly by cellular activation via FcγRs, which is enhanced in individuals with more activating variants. In the other diseases (SLE and RA), IgG does not seem to cause harm via cellular activation via FcγRs. Interestingly, the diseases in which the more activating variants are associated with susceptibility, are also the diseases in which IVIg is an effective therapy (KD and ITP), whereas IVIg is of no value in RA, Sjögren syndrome, systemic sclerosis and is possibly beneficial in SLE but this has not been well studied (162, 163), also suggesting a difference in the pathophysiological contribution of FcγRs in these diseases. An explanation for this finding may be that IVIg blocks activating FcγRs, which is beneficial in diseases in which these activating FcγRs are directly involved in pathophysiology, whereas in diseases in which activation of FcγRs does not play a role, blockade of FcγRs is not important.

Altogether, *FCGR2/3* association studies suggest that the pathophysiological mechanisms leading to SLE, RA, and Sjögren syndrome may be fundamentally different from the mechanisms leading to KD and ITP, a fact that is supported by the observation that SLE, RA and Sjögren syndrome occur much more frequently in women than in men, whereas in KD and ITP there is a slight predisposition in males.

Concluding, knowledge of *FCGR2/3* genetic variation in autoinflammatory and autoimmune diseases may increase our knowledge on the pathophysiology of these complicated and multifactorial diseases, and may be related to effectiveness of IVIg therapy.

FCGR2/3 GENETIC VARIATION AND PERSONALIZED MEDICINE

Autoimmunity and Transplant

Perhaps the most clinically useful application of genotyping *FCGR2/3* genetic variation could be predicting response to therapy, and *FCGR2/3* polymorphisms could be of potential value in personalized medicine. There is clinical data for Kawasaki disease, childhood ITP and SLE that correlated disease outcomes as well as response to treatment are associated with *FCGR2/3* variants as well as copy number variation of CNR1 (**Table 3**). This suggests that it may be possible to use genetic variants to determine prognosis and potentially guide treatment decisions. A key step toward their use would be external validation as well as an investigation of their integration with existing clinical prognostic scores.

Similar to autoimmunity, some data have suggested that patients with the higher-affinity *FCGR3A*-p.176Val allele show enhanced B cell deletion after rituximab treatment during liver transplant setting (**Table 3**). On the other hand, these patients

TABLE 3 | Opportunities to use *FCGR2/3* locus genotyping in personalized medicine: polymorphisms and copy number variation.

Domain	Disease setting	Clinical setting	Association with *FCGR2/3* genetic variation	References
Autoimmune	ITP	Prognosis	2B.4 and *FCGR2C*-ORF correlate are associated with transient disease; CNR1 deletion is associated with chronic disease	(65)
Autoimmune	ITP	Treatment	2B.4 and *FCGR2C*-ORF correlate with favorable IVIg response	(65)
Autoimmune	ITP	Treatment	*FCGR3A*-p.176Val/Val is associated with favorable response to rituximab	(164)
Autoimmune	Kawasaki disease	Treatment	2B.4 correlates with favorable IVIg response	(153)
Autoimmune	SLE	Prognosis	2B.4 shows lower rate of lupus nephritis	(11)
Autoimmune	SLE	Prognosis	CNR1 deletion is associated with lupus nephritis	(158)
Autoimmune	Rheumatoid arthritis	Treatment	*FCGR3A*-p.176Val allele confers improved response to rituximab treatment (meta-analysis, 3 studies)	(165)
Autoimmune	Rheumatoid arthritis	Treatment	*FCGR2A*-p.166Arg allele is associated with favorable response to adalimumab	(166)
Cancer	Breast cancer	Treatment	*FCGR2A*-p.166His/His and *FCGR3A*-p.176Val/Val have higher response and progression-free survival with trastuzumab in HER2-positive metastatic disease	(167)
Cancer	Breast cancer	Treatment	*FCGR2A*-p.166His/His shows better pathological response and progression-free survival after trastuzumab in HER2-positive disease	(168)
Cancer	Breast cancer	Treatment	*FCGR3A*-p.176Val/Val shows improved patient outcomes and benefit from trastuzumab	(169)
Cancer	Breast cancer	Treatment	No difference after trastuzumab observed with *FCGR2A*-p.166 and *FCGR3A*-p.176 variants	(170)
Cancer	Lymphoma	Treatment	*FCGR3A*-p.176Val/Val showed better response rates to rituximab	(171)
Cancer	Lymphoma	Treatment	*FCGR3A*-p.176Val/Val showed better response rates and progression-free survival to rituximab	(172)
Cancer	Lymphoma	Treatment	Carriers of *FCGR3A*-p.176Val allele showed better response rates	(173)
Cancer	Lymphoma	Treatment	Carriers of *FCGR3A*- p.176Val allele showed better response rates	(174)
Cancer	Lymphoma	Treatment	No difference in response to rituximab with *FCGR2A*-p.166 and *FCGR3A*-p.176 variants	(175)
Cancer	Lymphoma	Treatment	No difference in response to rituximab with *FCGR2A*- p.166 and *FCGR3A*-p.176 variants	(176)
Cancer	Lymphoma	Treatment	No difference in response to rituximab with *FCGR2A*- p.166 and *FCGR3A*-p.176 variants	(177)
Cancer	CLL	Treatment	No difference in response to rituximab with *FCGR2A*- p.166 and *FCGR3A*-p.176 variants	(178)
Cancer	Colorectal carcinoma	Treatment	*FCGR2A*-p.166His/His and *FCGR3A*-p.176Val/Val show longer progression-free survival after cetuximab	(179)
Cancer	Colorectal carcinoma	Treatment	*FCGR2A*-p.166His and *FCGR3A*-p.176Val alleles show increased response rates and stable disease after cetuximab	(180)
Cancer	Colorectal carcinoma	Treatment	*FCGR2A*-p.166His/His and *FCGR3A*-p.176Phe/Phe* showed higher progression-free surival rates after cetuximab	(181)
Cancer	Colorectal carcinoma	Treatment	*FCGR3A*-p.176Phe/Phe* showed higher survival rates after cetuximab	(182)
Cancer	Colorectal carcinoma	Treatment	*FCGR3A*-p.176Val/Val and p.176Val/Phe show higher progression-free survival than p.176Phe/Phe after cetuximab	(183)
Cancer	Colorectal carcinoma	Treatment	*FCGR3A*-p.176Val/Val showed shorter* progression-free survival compared to p.176Val/Phe or p.176Phe/Phe	(184)
Cancer	Head and neck carcinoma	Treatment	*FCGR2A*-p.166His/His and *FCGR3A*-p.176Val/Val have longer progression-free survival with cetuximab	(185)
Transplant	Liver transplant	Treatment	After rituximab, *FCGR2A*-p.166His/His shows stronger B cell suppression, bacterial infections, and poor prognosis	(186)

CLL, chronic lymphocytic leukemia.
**Result is in contrary direction to other studies.*

may require additional immune protection with IVIg because of increased susceptibility to bacterial infections. These data suggest that *FCGR3A* genotyping could be used to potentially offer an alternative transplant immunosuppression regimenboth during and following transplantation to intervene early to prevent immunosuppression-related complications.

Cancer Immunotherapy

Association of *FCGR2/3* genetic variation has also been extensively evaluated in monoclonal antibody therapy in cancer patients. Antibodies directed against specific tumor antigens may help in eradicating cancer cells, and this takes place in part by cellular effector mechanisms mediated by FcγRs, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Thus, the efficacy of the antibody may be related to *FCGR2/3* genetic variation. MoAb therapy is costly, and since not all patients seem to benefit from it, predicting an individual patients' response could help to identify the patients likely to respond to this therapy. Extensive data points toward the fact that patients with higher-affinity *FCGR2A*-p.166His or *FCGR3A*-p.176Val alleles have an enhanced response to monoclonal antibody-mediated anti-cancer therapy amongst solid tumors of the breast, head and neck, colorectal carcinomas as well as lymphomas (**Table 3**). These data are supported by extensive molecular evidence. For example, the trastuzumab mediated ADCC of anti-HER2 breast cancer cells is enhanced with *FCGR2A*-p.166His and *FCGR3A*-p.176Val, compared to the other alleles (167), and in case of neutrophil-mediated ADCC for *FCGR2A*-p.166His (187). The same has been observed with NK cell rituximab-mediated ADCC to Daudi (lymphoma) cells in *FCGR3A*-p.176Val individuals (23). Collectively, these data show on a laboratory and clinical level that individuals with *FCGR2A*-p.166His and *FCGR3A*-p.176Val have enhanced responses to antibody-mediated cancer immunotherapy and genotyping may be useful to stratify treatment regimens and potentially adjust dosing to prevent side effects. Notably, some studies have shown discordant results that are in contrast with these findings (**Table 3**).

Currently, the variation in results do not yet allow a strategy to justify individualized treatments to be on the basis of *FCGR2/3* polymorphisms (188). When clinical use of afucosylated IgG antibodies with increased affinity for FcγRIIIA could be combined with *FCGR2/3* genotyping, the correlation with efficacy of cancer therapy may be enhanced. A major drawback of most current genotyping studies is the fact that only two SNPs are analyzed, whereas other SNPs at the locus also potentially influence treatment response rates, and the SNPs are in LD with each other. Analysis of all the SNPs and CNV, and analysis as extended haplotypes across the locus may be more useful; the complexity of the locus requires a more comprehensive assessment that includes determination of gene copy numbers, as well as classic *FCGR2C*-ORF haplotype.

CONCLUSION

The *FCGR2/3* locus is a complex genetic locus with many functional genetic variants in intricate linkage. It holds many disease associations which are different, sometimes with opposite effects, between various autoimmune and autoinflammatory diseases, which may inform us on fundamental differences in pathophysiologic mechanisms. Furthermore, the locus is promising in view of genetic prediction of efficacy of therapy, especially immunotherapy in cancer, although this is currently not yet feasible. Given the complexity of the locus and inaccuracies in the current databases holding reference sequences, research on the locus could benefit from a thorough genetic analysis that sequences through the entire region and can help to establish a correct and proper reference. Such an approach has recently been explored for *FCGR3A* using long-range sequencing with Nanopore MinION technology, and allowed a complete investigation of polymorphic sites within the gene (189). In any case, to use the full potential of genetic variation at the *FCGR2/3* locus, a comprehensive analysis of all SNPs and CNVs together is warranted.

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REVIEW
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The Complex Association of FcγRIIb With Autoimmune Susceptibility

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FcγRIIb is the only inhibitory Fc receptor and controls many aspects of immune and inflammatory responses. The observation 19 years ago that *FcγRIIb*$^{-/-}$ mice generated by gene targeting in 129 derived ES cells developed severe lupus like disease when backcrossed more than 7 generations into C57BL/6 background initiated extensive research on the functional understanding of this strong autoimmune phenotype. The genomic region in the distal part of Chr1 both in human and mice in which the *FcγR* gene cluster is located shows a high level of complexity in relation to the susceptibility to SLE. Specific haplotypes of closely linked genes including the *FcγRIIb* and *Slamf* genes are associated with increased susceptibility to SLE both in mice and human. Using forward and reverse genetic approaches including in human GWAS and in mice congenic strains, KO mice (germline and cell type specific, on different genetic background), knockin mice, overexpressing transgenic mice combined with immunological models such as adoptive transfer of B cells from Ig transgenic mice the involved genes and the causal mutations and their associated functional alterations were analyzed. In this review the results of this 19 years extensive research are discussed with a focus on (genetically modified) mouse models.

Keywords: SLE, systemic lupus erythematosus, autoimmue disease, mouse model, Fcgamma receptor IIB, reverse genetics

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INTRODUCTION

Antibodies (Ab) form immune complexes (IC) with their cognate antigen (Ag). IgG-ICs are potent activators of the immune system via cross-linking of receptors for the Fc part of IgG, FcγR, mainly expressed on the surface of cells of the innate immune system.

FcγRs belong to the Ig supergene family of leukocyte FcR and are transmembrane glycoproteins containing a ligand-binding α subunit with two or three extracellular Ig-like domains, a transmembrane and a cytoplasmic domain. In mice, the high-affinity FcγRI, binding monomeric IgG, and the low-affinity receptors for complexed IgG, FcγRIII, and FcγRIV are activating receptors. The α subunits of the activating receptors form a multi-subunit complex with a dimer of the common γ-chain (FcRγ) (1, 2) with an immunoreceptor tyrosine-based activation motif (ITAM). Cross-linking activating FcRs by IC initiates signal transduction via recruitment and subsequent activation of intracellular tyrosine kinases (3), switching on a large variety of effector mechanisms activating inflammatory cascades.

In humans, there are four activating FCGRs. The high-affinity FCGR1 (CD64) and the low-affinity FCGR3A (CD16A) are associated with the common γ chain whereas the low-affinity FCGR2A (CD32A), containing an ITAM in its cytoplasmic domain, and the low-affinity FCGR3B (CD16B), with a glycosylphosphatidylinositol (GPI) anchor, are single-chain receptors. All human

FCGR genes are clustered at the distal end of Chr1, a region associated with susceptibility to autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (4). In mice the FcγRII, -III, and -IV genes are clustered at the distal end of Chr1, a region orthologous with SLE associated genomic intervals on human Chr1 and associated also with susceptibility to autoimmune disease (Lupus-like disease). *Fcγ RI* is located on Chr3 due to a translocation during evolution after mouse and human had diverged.

In both humans and mice, the activating FcγRs are counterbalanced by one inhibitory single-chain low-affinity receptor FcγRIIb (FCGR2B or CD32B) with an inhibitory motif named immunoreceptor tyrosine-based inhibition motif (ITIM) within its cytoplasmic domain. In addition, co-engagement of FcγRIIb and the ITAM containing B-cell receptor (BCR) on B cells forms an important negative feedback mechanism to control antibody production. This regulatory mechanism of cellular activation by the ITAM-ITIM motif pair, observed originally with FcγR, has been described for many other receptors in the immune system e.g., T cell receptors and B cell receptors (5, 6). This review focuses on the important but still puzzling immune regulatory role of the inhibitory FcγRIIb and the complex association of its impaired function with autoimmunity as studied extensively in mice.

GENERAL CHARACTERISTICS OF FcγRIIb

Isoforms

In humans and mice, there are two membrane-bound isoforms of FcγRIIb identified: FcγRIIb1 and b2 (7) resulting from alternative splicing. The cytoplasmic domain is encoded by three exons whose 5′ exon encodes a 47 amino acid motif that prevents coated pit localization, which inhibits FcγRIIb mediated endocytosis of soluble immune complexes. This exon is present in the mRNA that encodes the b1 isoform, the only isoform expressed on B cells, but absent in the mRNA that encodes the b2 isoform (8, 9) expressed on most innate immune cells. The ITIM dependent inhibition of cell activation is the same for both isoforms. Therefore, the name FcγRIIb is used in this review without making a distinction between the b1 and the b2 isoform.

Expression

In mice FcγRIIb is expressed on all innate immune cells and is the only FcγR expressed on B cells, including pre-, pro-, and mature B cells, memory B cells, plasma cells (10, 11) and B1 cells (12). Unlike many other B cell surface receptors, expression of FcgRIIb is not downregulated during plasma cell differentiation (10). FcγRIIb expression is modulated on different B cell subsets (11) and increases when the B cells become activated (11, 13). T cells do not intrinsically express FcγRs (14). However, it has been reported that expression of FcγRIIb but not any other FcγR, is upregulated in memory $CD8^+$ T cells after *Listeria monocytogenes* infection and tempers the function of these cells *in vivo* (15). Guilliams et al. showed that according to the microarray expression values extracted from public data sets the mRNA expression of FcγRIIb in mice is from high to low as follows: Inflammatory macrophages (Mφ), $Ly6C^{hi}$ classical monocyte, inflammatory monocyte-derived dendritic cell (moDC), lung $CD11b^+$ conventional or classical DC (cDC), $Ly6C^{lo}$ patrolling monocyte, alveolar Mφ, follicular B cell, GC B cell, skin-draining lymph node $CD11b^+$ cDC, spleen $CD8^+XCR1^+$ cDC, spleen plasmacytoid DC (pDC), spleen $CD11b^+$ cDC, neutrophils, spleen Mφ, and NK cells (16). The overall FcγRIIb expression pattern is similar in mouse and human. In mouse cDCs the relatively low expression of FcγRIIb is higher than that of any activating FcγR.

FcγRIIb expression, relative to that of activating FcγRs, is tightly regulated. In mice, C5a rapidly down-regulates FcγRIIb on alveolar Mφ and upregulates FcγRIII on these cells (17, 18). IL-4 downregulates FcγRIIb expression on mouse activated B cells (13, 19). IFNγ increases FcγRIIb expression on B cells (19) and increases the expression of activating FcγR on myeloid effector cells in mice. In humans the Th2 cytokines IL-4, IL-10, and TGF-β increase FCGR2B expression and decrease activating FCGR expression on myeloid cells (20–22) whereas IFNγ decreases FCGR2B expression on these cells and increases activating FCGR expression (23).

FcγRIIb is also expressed on non-hematopoietic cells. Its expression is induced on FDC upon antigen stimulation (24). It has been calculated that almost 70% of total mouse body FcγRIIb is expressed on liver sinusoidal endothelial cells (LSEC) (25, 26). On mouse glomerular mesangial cells, TNFα/IL-1β upregulates FcγRIIb expression whereas IFNγ downregulates FcγRIIb expression and upregulates the activating FcγR (27).

Cellular Function

Co-aggregation of the inhibiting ITIM containing FcγRIIb with activating ITAM containing FcRs results in the recruitment of the inositol polyphosphate-5-phosphatase SHIP1 that counteracts the signals mediated by activating FcRs (3, 28). Therefore, FcγRIIb has a strong regulatory role in all the processes in which activating FcγR are involved. The ratio between activating and inhibiting signals determines the outcome of the cellular response to IgG-ICs. This ratio depends mainly on the following factors: (a) the relative affinities of the different antibody isotypes involved for the different FcγR, (b) the level of opsonization, and (c) the relative expression level of inhibitory and activating FcγR, which is partially determined by the cytokine milieu. The binding of FcγRIIb for IgG-IC is strongest for IgG1 and weakest for IgG2a. So, FcγRIIb expression has the highest impact on IgG1-IC. In addition, FcγRIIb can inhibit complement-mediated inflammation when co-engaged with Dectin-1 by galactosylated IgG1-ICs (29) indicating that its immune-modulatory function in the efferent response is not restricted to the regulation of activating FcγRs.

In B cells co-crosslinking of the BCR and FcγRIIb results in the inhibition of activation, proliferation, Ag internalization and Ab secretion (30–32). Moreover, *in vitro* studies have shown that FcγRIIb on B cells can induce apoptosis upon clustering (10, 12, 28, 33, 34).

FcγRIIb can also function as an endocytic receptor of small ICs. The endocytic properties of FcγRIIb depend on the presence of a di-leucine motif in the intracellular domain (8) and are independent of the ITIM.

Role in Different Tissues and Cell Types

Myeloid Effector Cells

In the efferent phase, FcγRIIb sets a threshold for the activation by IgG-IC of myeloid effector cells, e.g., monocytes, Mφs, and neutrophils. Crosslinking of activating FcγR by IgG-ICs induces effector mechanisms of these cells e.g., soluble IC clearance, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), the release of inflammatory mediators, degranulation, superoxide production, enhancement of Ag presentation, and cell maturation and proliferation. This includes also the regulation of high-affinity IgE receptor-mediated mast cell activation (35).

Lupus-prone (NZBxNZW)F1 mice deficient for the FcR γ chain, lacking functional activating FcγR, do not develop IC-mediated severe glomerulonephritis (GN), despite high autoantibody titers (36). This suggests that FcγR play a dominant role in the efferent phase of Ab-driven diseases including lupus-like disease and therefore FcγRIIb might have a strong protective role in such a disease. In addition, FcγRIIb might also inhibit an ongoing auto-Ab response by suppressing the activating FcγR dependent, IgG-IC-triggered release of inflammatory mediators and other immune regulatory molecules by myeloid effector cells.

Dendritic Cells

DCs are central regulators of immunity determining whether tolerance is induced, or an effective adaptive immune response is generated, bridging innate and adaptive immunity (37–39). DCs have the unique capacity to take up exogenous Ag via a variety of mechanisms and surface molecules, including FcγR, and subsequently process and present the Ag-derived peptides in their MHC molecules to prime naïve T cells. Three main subsets of DCs can be recognized, cDC, moDC and pDC. Their ontogeny and functions have been reviewed extensively (40, 41).

A series of observations suggest that FcγR on cDCs and moDCs can play a role in priming and regulation of adaptive immunity (16). Ag-specific IgG enhances Ab responses to soluble protein Ag via activating FcγRs, probably by increasing Ag presentation by dendritic cells to Th cells (42). Many laboratories have shown that soluble IgG-ICs strongly enhance cross-presentation by using either *in vitro* assays (43–45), or *in vivo* assays with *in vitro* loaded DCs from WT and FcγR KO mice (46–50). Signaling through the activating FcγRs results in lysosomal targeting of the Ag and importantly activation and maturation of the DCs (44), required for their migration to the lymph node and their presentation of Ag-derived peptides in MHC class I to $CD8^+$ T cells (49, 51). In mouse bone marrow-derived DCs (BMDCs), activating FcγRs modulate the expression of many genes, associated with T cell response induction, upon crosslinking by IgG-ICs. This is strongly regulated by FcγRIIb, setting a threshold for DC activation and maturation (52). *FcγRIIb*$^{-/-}$ mice showed an increased upregulation of costimulatory molecules, resulting in an enhanced capacity to generate antigen-specific T cell responses upon injection of IgG-ICs (52–54). However, *in vivo*, in mice, the role of FcγR in the presentation of soluble IgG-IC derived Ag is redundant (55, 56). In mice, cDCs consist of two main subsets, type 1 cDC or cDC1 and Type 2 cDC or cDC2 (41). *In vivo* IgG-IC improve strongly cross-presentation of the cDC2 but not the cDC1 DCs. Only cDC2 mediated cross-presentation is FcγR dependent (57). Moreover, FcγRs are dispensable for the *in vivo* uptake of IgG-IC by cDC1 and cDC2 (56, 57). The *in vivo* cross-presentation of IgG-IC derived Ag by cDC1 is completely and by cDC2 partially dependent on C1q (56).

Because it has been shown that treatment with FCGR2B blocking antibodies results in spontaneous maturation of human DCs (58) it has been hypothesized that FCGR2B does not only regulate DC activation but also actively prevents unwanted spontaneous DC maturation by small amounts of circulating IC present in serum under non-inflammatory steady-state conditions (2).

IgG-ICs endocytosed by activating FcγR on DCs ends up in a degradative Lamp-1 positive compartment where it is slowly degraded into peptides (59). In contrast, antigen, endocytosed in the periphery via FcγRIIb on DCs, enters preferentially in a non-degradative Lamp-1 negative intracellular vesicular compartment, that recycles to the cell surface to transfer the native antigen via interaction with the BCR to B cells in the lymphoid organs. This indicates that DCs, migrating into extrafollicular areas (60) and the splenic marginal zones (MZ) (61), are not only important for the production of B cell activating components but also for the delivery of Ag to the BCR (62).

The question is whether in an autoimmune disease self-antigen containing IgG-IC can trigger DCs to promote autoreactive immune responses by presenting autoantigens or to release B and T cell activating cytokines and other stimulating factors breaking tolerance and whether FcγRIIb on DCs negatively regulates these processes. That is any way at a stage of the disease that some autoantibodies are already produced.

pDCs produce type I IFN in response to viral nucleic acids sensed through TLR7 and TLR9 (63, 64). Their main function is to control tolerance in the steady state (65, 66). Mouse pDCs express exclusively FcγRIIb (67). Conflicting results have been published regarding FcγRIIb facilitated T cell priming by mouse pDCs (56, 67, 68). *In vitro* uptake of IgG-ICs by mouse pDC is FcγRIIb dependent but does not promote Ag presentation to T cells (67), similarly to what has been shown with FcγRIIb mediated IC uptake in cDCs (62). In contrast, it has been reported that subcutaneous (s.c.) injection of *in vitro* IgG1-IC loaded pDCs induces strong Ag-specific $CD4^+$ and $CD8^+$ T cell responses although with lower efficiency than cDCs. The IgG1-IC-loaded pDC mainly promoted a Th2/tolerogenic environment *in vivo* (68). Human pDCs express besides low levels of FCGR2B, the activating FCGR2A and FCGR3B (16) and show FCGR2A dependent IgG enhanced Ag presentation to T cells (69). SLE patients have circulating ICs, containing small nuclear RNA and anti-small nuclear RNA IgG. pDCs can acquire such IC via FCGR mediated uptake resulting in stimulation of TLR7 and 8 and production of IFNα (70), a cytokine that is believed to play a central role in SLE pathogenesis (71). However, this requires FCGR2A and not FCGR2B (72). Therefore, it is

unlikely that such a pathogenic process plays a role in lupus-like disease in mice.

B Cells and FDC

Primary B cells, developed and selected in the bone marrow, are recruited into GCs within the spleen and lymph nodes to undergo affinity maturation by Somatic Hypermutation (SHM). Three main mechanisms maintain self-tolerance in the primary B cell repertoire: central clonal deletion, receptor editing, clonal anergy induction (73). The first two effectively remove autoreactive B cells from the system. Clonal anergy occurs when self-reactive B cells interact with a self-Ag with relatively low avidity. The result is that BCR signaling is desensitized because of chronic exposure to self-antigens (74, 75) and differentiation into plasma cells is suppressed (76) resulting in the maintenance of anergic B cells with the potential to produce auto-Abs which can be recruited into GC (77). Anergic B cells can get T help if their BCR cross-reacts with foreign Ag but because of impaired BCR signaling FAS-mediated apoptosis is induced. However, extensive cross-linking by a foreign antigen can overcome the attenuated BCR signaling in anergic B cells inhibiting apoptosis (74). Autoreactive primary B cells can escape negative selection because of "clonal ignorance" when self-reactive B cells cannot encounter their self-Ag because it is hidden inside the cell. Development, responsiveness, and lifespan of ignorant cells is normal (76, 78, 79). The lack of T cell help after Ag contact induces apoptosis in ignorant self-reactive B cells in the periphery. However, it is striking that many auto-Abs are directed against intracellular Ags such as DNA. Therefore, it has been suggested that ignorant self-reactive B cells might be important for the development of SLE (77). So, the GC has to deal with three types of potential autoreactive B cells: anergic and ignorant, both recruited, and newly generated by somatic hypermutation in the GC reaction. Several mechanisms are in place in the GC to avoid the development of auto-Ab producing plasma cells. A very high concentration of self-Ag in the GC either overrules the binding of the BCR to foreign Ag presented by the FDC and apoptosis is induced, because of the lack of additional signals provided by the FDC (80), or/and blocks presentation of foreign Ag to follicular helper T cells (T_{FH}), whose survival signals are required. Alternatively, self-reactive B cells can be maintained temporarily until their self-reactivity is abrogated by somatic hypermutation (SHM) (81). Ignorant self-reactive primary B cells, activated by cross-reactive foreign Ag, can enter the GC to get T_{FH} help (82) and subsequently, receptor editing by SHM can destroy self-recognition and improve specificity for foreign antigen. However, this appears not sufficient to prevent that autoreactive B cells escape negative selection in the GC and enter the AFC (antibody-forming cell) pathway. More downstream tolerance checkpoints are required.

In the GC Ag is presented to B cells on the cell surface of FDC, mainly in the form of CR1/2 bound C3d-coated ICs. FcγRIIb is expressed on both the GC B cell and the FDC. Although FcγRIIb is upregulated on FDC in GC compared to non-GC FDC, its expression is relatively low compared to CR2 expression. Therefore, it is unlikely that FcγRIIb plays a role in the capture and presentation of Ag early on in the GC response (83). It is unclear how a GC B cell becomes activated, because binding of its BCR to the Ag, within the FDC bound ICs, will also crosslink FcγRIIb on that B cell. It has been suggested that FcγRIIb expression on FDC competes with FcγRIIb expression on GC B cells by binding most of the Fc domains in the ICs (84). The outcome of co-engagement of BCR and FcγRIIb by ICs bound to FDC in GC might be dependent on the balance between concurrent activating and inhibiting signals, leading to stimulatory, inhibitory, or apoptotic responses (33, 85, 86). FcγRIIb might set a threshold for B-cell activation, that enables the selection of B cells with a BCR with sufficiently high affinity, to become activated. B cells with BCRs that have lost their affinity for the presented Ag during the process of affinity maturation by SHM will get only signals via crosslinking of FcγRIIb, which could result in induction of apoptosis as has been demonstrated *in vitro* (28, 33, 87, 88). In conclusion, the inhibitory FcγRIIb would be an important checkpoint for the deletion of potentially autoreactive B cells in the GC.

An additional apoptosis inducing mechanism in the bone marrow might also contribute to the control of autoreactive B cells (10). Long-lived plasma cells persist in the bone marrow. To provide room to newly generated plasma cells that migrate to the bone marrow after a new infection has occurred, a restricted number of plasma cells in the bone marrow has to be eliminated. Based on observations *in vitro* and *in vivo* in mice it has been hypothesized that plasma cells (which intrinsically lack BCR expression) are killed by apoptosis, induced by cross-linking of FcγRIIb highly expressed on these cells (10).

Non-immune Cells

On LSEC FcγRIIb might function as an endocytic scavenger receptor removing small IgG-IC from circulation to prevent systemic IC triggered inflammation (25). FcγRIIb on renal mesangial cells might protect against IgG-IC induced inflammation in the kidney (89). Both mechanisms might protect against the pathogenesis of IC-driven autoimmune diseases such as glomerulonephritis in SLE in the efferent phase. Because of the lack of an endothelial cell-specific Cre expressing strain that is not transcriptionally active during early hematopoiesis, required to generate endothelium-specific FcγRIIb deficient mice, the specific role of FcγRIIb on LSEC should be studied by applying transplantation of bone marrow from WT mice into lethally irradiated *Fcγ RIIb* KO mice.

FORWARD GENETICS: ASSOCIATION OF AUTOIMMUNITY AND FcγRIIb POLYMORPHISM

In Mice

The association between autoimmunity and *Fcγ RIIb* polymorphism is extensively studied in NZW and NZB inbred stains. NZB mice show limited autoimmunity (90) while NZW mice are not autoimmune although their B cells have intrinsic defects sufficient to break tolerance to nuclear antigens (91, 92). However, the (NZBxNZW)F1 offspring of an accidental cross between NZW and NZB mice (93) showed a severe lupus-like

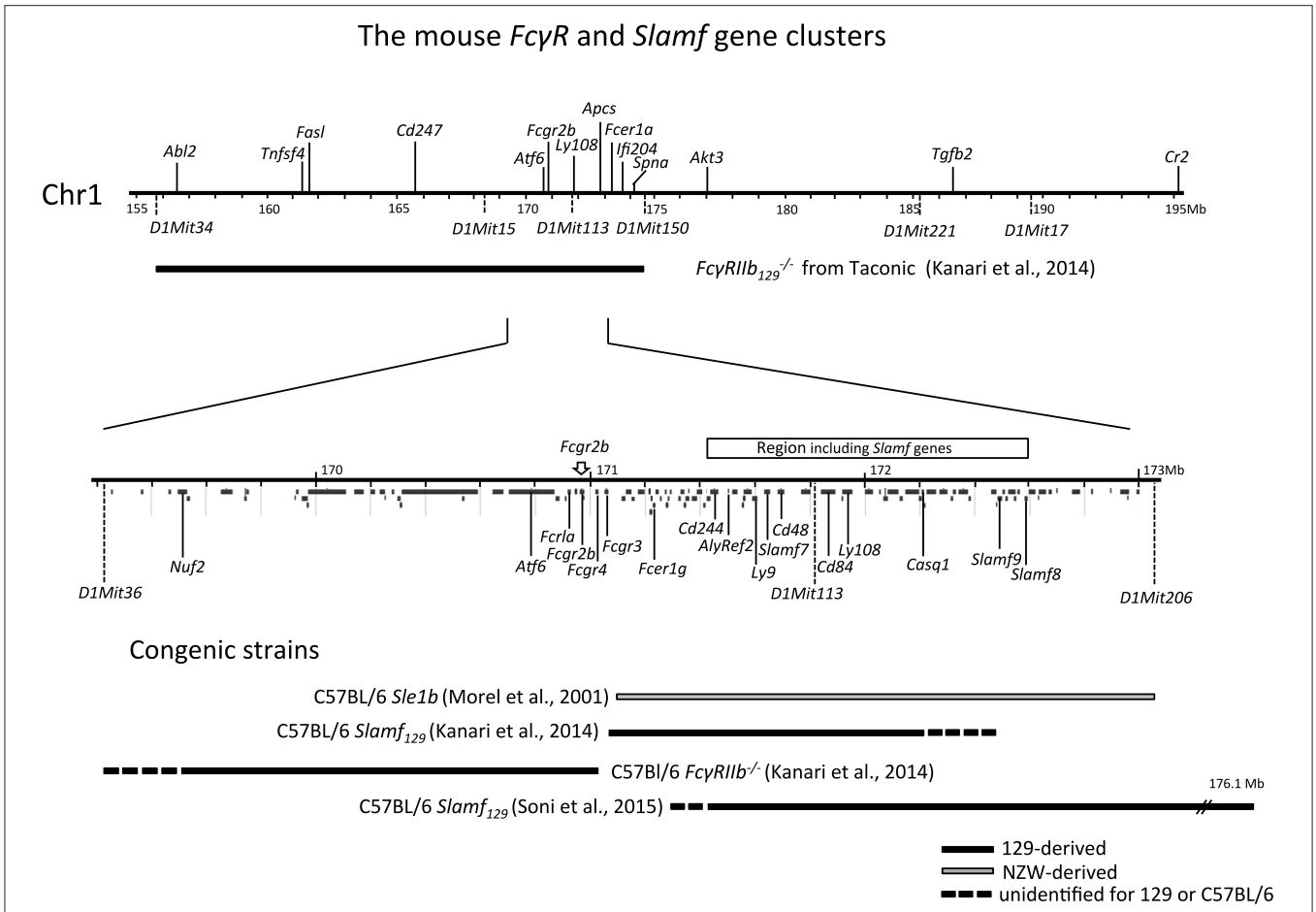

FIGURE 1 | Physical map of the sub-telomeric region of mouse chromosome 1. The upper horizontal line represents a 40 Mb genomic region including the 129 derived *FcγRIIb* flanking region present in the original $FγRIIB^{-/-}_{129}$ mouse, backcrossed more than 7 generations into C57BL/6 background. This *FcγRIIb* flanking region spans at a minimum the distance between the microsatellite markers *D1Mit34* and *D1Mit150* [horizontal black bar; (98)]. The lower horizontal line represents a magnification of the 3.8 Mb subregion located between microsatellite markers *D1Mit36* and *D1Mit206* showing a detailed map of the *FcγR* and *Slam* family gene clusters within this region. In addition, below the line, the location of all other coding genes in this region is shown according to the NCBI database. At the bottom, the congenic fragments present in the different $FcγRIIb^{-/-}$ and $Slamf_{129}$ C57BL/6 congenic strains, described in the text, are depicted as horizontal bars.

phenotype characterized by a gender-bias, expansion of activated B and $CD4^+$ T cells, splenomegaly, elevated serum ANA and IC-mediated GN causing renal failure and premature death at 10–12 months of age (94). By backcrossing (NZBxNZW)F1xNZW followed by brother-sister mating the NZM2410 recombinant inbred strain with a homozygous genome was generated (95–97). In this mouse four SLE susceptibility loci, *Sle1-4*, have been identified on different chromosomes. *Sle1* is located on the telomeric region of Chr1 syntenic to human 1q23 that has shown strong linkage to SLE susceptibility in all human studies. The *FcγR* gene cluster maps in this region (**Figure 1**) and is from NZW origin in NZM2410 mice. From the NZM2410 strain, C57BL/6 strains have been developed congenic for a single SLE susceptibility locus. The presence of *Sle1* appeared to be sufficient to break tolerance in C57BL/6 mice and to drive the production of high titers of anti-chromatin ANAs with a selective Ab reactivity to H2A/H2B/DNA sub-nucleosomes (99, 100). Importantly, this step appears to be necessary for the induction of disease (100) making *Sle1* a key locus in the initiation of SLE. Transplantation of hematopoietic stem cells from C57BL/6 *Sle1* congenic mice into C57BL/6 recipient mice showed that *Sle1* causes independent B and T cell-intrinsic effects on the B cell response (101, 102).

Three *FcγRIIb* haplotypes [numbered I-III according to Jiang et al. (103), **Table 1**] have been recognized in inbred strains of mice and wild mice with variation in the promoter region and intron 3 (**Table 1**). Haplotype I with 2 deletions in the promoter region and one in intron 3 is found in autoimmune-prone strains and most wild mice and is associated with decreased expression of FcγRIIb on Mφ, activated B cells and GC B cells (11, 103–105). By using C7BL/6 congenic strains with the NZW (106) and NZB (107) allelic variants of *FcγRIIb* the effect of the deletions in haplotype I and II on B cell expression was studied. When immunized with KLH, FcγRIIb expression on splenic non-GC B

TABLE 1 | Allelic variants of mouse *FcγRIIb* gene and their association with impaired expression and autoimmune disease susceptibility.

Haplotype	Mouse strain	Genetic variation	Phenotype
I	NZB, BXSB, MRL, NOD, Wild mice 129	13 bp 5′ deletion in promoter 3 bp 3′ deletion in promoter 4 bp 5′ deletion in intron 3	Decreased expression on Mφ and activated and GC B cells. Autoimmune-prone (except 129)
II	NZW, SWR, SJL	4 bp 5′ deletion in intron 3 24 b 3′ deletion in intron 3	Decreased expression on GC B cells. Potential to accelerate autoimmunity
III	C57BL/6, BALB/c, DBA	No deletions	Not autoimmune

cells was high and similar in C7BL/6 and C57BL/6 $Fc\gamma RIIb_{NZB}$ congenic mice. In contrast, the expression on activated GC B cells was markedly down-regulated in C57BL/6 congenic $Fc\gamma RIIb_{NZB}$ mice and up-regulated in control C57BL/6 mice, in comparison with the expression levels on non-GC B cells. The downregulation of FcγRIIb expression on activated GC B cells was associated with an increase of IgG anti-KLH Ab titers. C57BL/6 $FcgRIIb_{NZB}$ congenic mice also showed lower FcγRIIb expression on Mφ compared with WT C57BL/6 mice (107). In a C57BL/6 knockin (KI) mouse model of the 5' region of the haplotype I $Fc\gamma RIIb$ gene ($Fc\gamma RIIb_{NZB}$), FcγRIIb failed to be upregulated on activated and GC B cells resulting in enhanced early GC responses and low auto-Ab production without kidney disease as discussed later in more detail (11).

As mentioned earlier, *in vitro* cross-linking of FcγRIIb on B cells from C57BL/6 mice can induce apoptosis. However, plasma cells from autoimmune-prone NZB or MRL mice could not be killed *in vitro* by FcγRIIb cross-linking because of too little expression of the receptor (10). This might partially explain why these autoimmune-prone mice have larger numbers of plasma cells and might contribute to the autoimmune phenotype of these mice.

Similarly, to the $Fc\gamma RIIb_{NZB}$ allele, the $Fc\gamma RIIb_{NZW}$ allele in the C57BL/6 *Sle1* congenic strain did not upregulate its expression on GC B cells and plasma cells, as did the C57BL/6 allele, when immunized with SRBCs. However, in the absence of its *Sle1* flanking regions, $Fc\gamma RIIb_{NZW}$ did not induce an autoimmune phenotype but was associated with an increased number of class-switched plasma cells (108). This might indicate that the decreased expression of the $Fc\gamma RIIb_{NZW}$ allele is not sufficient for the development of autoreactive B cells but can result in the increase of the number of autoreactive B cells, induced by other lupus-susceptibility loci, by enhancing the production of class-switched plasma cells. This suggests that the $Fc\gamma RIIb_{NZB}$ (haplotype I) allele has a stronger impact on susceptibility to autoimmunity than the $Fc\gamma RIIb_{NZW}$ (haplotype II) allele (**Figure 2**). However, in one study comparing the phenotypes of C57BL/6 strains congenic for different intervals of the *Nba2* locus, a region on Chr1 of NZB mice corresponding to the $Sle1_{NZW}$ locus, $Fc\gamma RIIb_{NZB}$ was identified as an autoimmune susceptibility gene (114), in another it was not (115). *Sle1* can be divided in four non-overlapping sub-loci: *Sle1a, -b, -c*, and *-d. Sle1b* is far the most potent autoimmune susceptibility locus causing almost the same phenotype as the whole *Sle1* locus: gender-biased spontaneous loss of immune tolerance to chromatin, the production of high titers of IgG auto-Abs with a penetrance of 90% at 9 months of age and increase of total IgM and B7-2 expression on B cells (116). This suggests that *Sle1b* mainly affects B cells. The genomic location of *Sle1b* was determined by phenotypic analysis (e.g., ANA production) of a series of C57BL/6 congenic strains carrying truncated *Sle1* intervals. C57BL/6 congenic mice with an NZW derived genomic fragment, containing the $Fc\gamma R$ cluster, did not develop ANA whereas C57BL/6 mice, containing an adjacent 900 kb congenic NZW fragment expressing 24 genes including seven members of the highly polymorphic signaling lymphocytic activation molecules (*Slam*) cluster, did. This positions the $Fc\gamma R$ cluster just outside the *Sle1b* locus (117) and confirms previous observations that $Fc\gamma RIIb$ is located in between the *Sle1a* and *Sle1b* loci (113) (**Figure 1**). Together these data suggest that in C57BL/6 *Sle1* congenic mice the $Fc\gamma RIIb_{NZW}$ allele is not required for the development of an autoimmune phenotype, whereas the adjacent *Slam* cluster is. Because of these puzzling results, the questions remain why FcγRIIb is upregulated on GC B cells in non-autoimmune inbred strains such as C57BL/6 and BALB/c and why this is impaired in autoimmune-prone mouse strains and how does that contribute to the autoimmune phenotype of these mice.

Slam family (*Slamf*) member genes encode cell surface glycoproteins with extracellular binding domains that mediate stimulatory and/or inhibitory signaling via associations with members of the Slam-associated protein (SAP) family of signaling adaptors during cell-cell interactions between many hematopoietic cell types (118–120). They are the only genes within the *Sle1b* interval with obvious immunological functions (117). Most Slamf members act as self-ligand and are expressed on many lymphoid and myeloid cell subsets, platelets, and hematopoietic stem and progenitor cells. Slamf plays a role in the interaction of $CD4^+$ T cells with cognate B cells, recruitment and retention of T cells within the emerging GCs (121–123), long-lasting T cell:B-cell contact, optimal T_{FH} function, T cell activation (124, 125), stabilization of B–T cell conjugates and sustaining effective delivery of T cell help required for GC formation (126, 127).

The *Slamf* genes show extensive polymorphisms (117) but only two haplotypes of the *Slamf* locus have been identified in laboratory mouse strains. Haplotype 1 is represented by C57BL/6 and related strains and haplotype 2 by all autoimmune-prone mouse strains, as well as many non-autoimmune mouse strains including BALB/c and 129. The polymorphism in *Slamf* member *Ly108* affects the expression of two alternatively spliced isoforms, Ly108-1 and, Ly108-2, which differ exclusively in their cytoplasmic region (117). Ly108-1 is dominantly expressed in T and B lymphocytes of mice with haplotype 2, whereas Ly108-2 is dominantly expressed in T and B cells of mice with haplotype 1. Modulation of the BCR signaling by Ly108-1 results in the impaired negative selection of B cells (128). Overexpression of

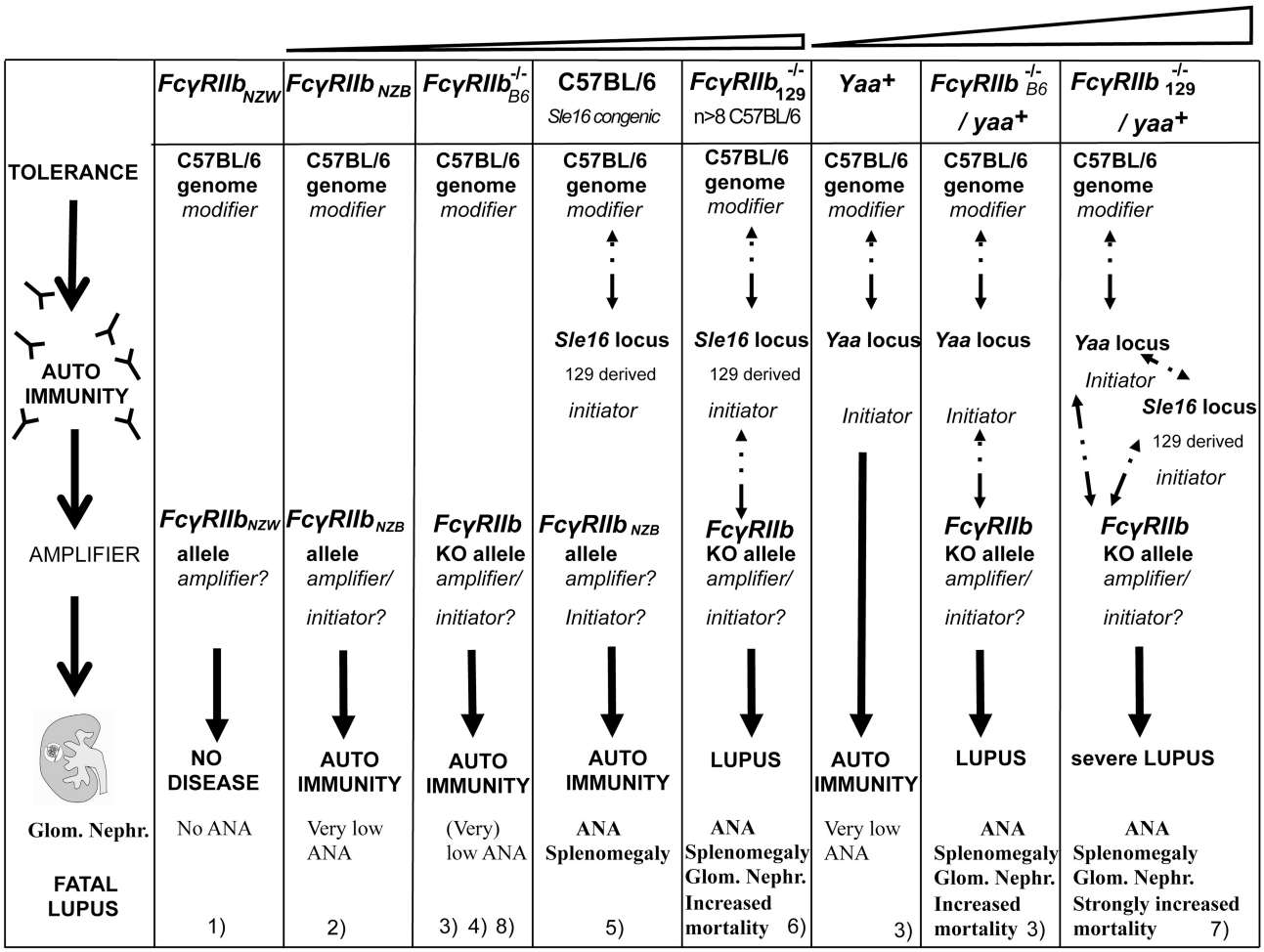

FIGURE 2 | Epistasis between the *FcγRIIb* KO alleles and the *Sle16 (Slam*$_{129}$*)* and *Yaa* autoimmune susceptibility loci resulting in lupus-like disease in C57BL/6 mice. Epistatic interactions are indicated as dotted arrows. The FcγRIIb flanking *Sle16* genomic region contains the autoimmunity associated *Slamf*$_{129}$ haplotype 2 gene cluster (see **Figure 1**). (1) Rahman et al. (108); (2) Espéli et al. (11); (3) Boross et al. (109); (4) Li et al. (110); (5) Bygrave et al. (111); (6) Bolland and Ravetch (112); (7) Bolland et al. (113); (8) Kanari et al. (98). The increasing severity of autoimmune disease in the different mouse models is depicted on top.

both C57BL/6 derived non-autoimmune Ly108 and CD84 Slamf members was required to restore tolerance in autoimmune-prone C57BL/6 *Sle1* congenic mice (129), indicating that polymorphism in both *Slamf* genes contributes to the autoimmune phenotype of C57BL/6 *Sle1* congenic mice.

In the NZM2410 model four NZW-derived SLE suppressor loci have been identified (130). The presence of such suppressor loci might explain why NZW and also129 and BALB/c mice do not develop autoimmune disease, although they carry the type 2 *Slamf* haplotype.

In Humans

The reported copy number variation (CNV) in human *FCGR* genes does not involve *FCGR2B* (131–134). A series of single nucleotide polymorphisms (SNPs) have been reported to be located both in the promoter and the encoding region of the human *FCGR2B* gene (135). Two SNPs are located in the promoter region at nucleotide positions−386 and −120 (−386G>C; *rs3219018* and −120A>T; *rs34701572*) (136) resulting in four haplotypes:−386G−120T (named *FCGR2B.1*), −386C−120T (*FCGR2B.2*),−386G−120A (*FCGR2B.3*), and −386C−120A (*FCGR2B.4*). The rare *FCGR2B.4* haplotype increased the transcription of *FCGR2B in vitro* and resulted in increased FCGR2B expression on EBV transformed B cells and primary B cells (137) and myeloid cells (138), compared to the more frequent *FCGR2B.1* haplotype. However, independently, others have shown that homozygosity of the −386C genotype decreases the transcription and surface expression of FCGR2B in peripheral B cells compared to the −386G homozygote genotype (139). Up till now, there is no explanation for these contradictory results.

In the transmembrane encoding fifth exon a non-synonymous C to T transition was identified, *rs1050501*, resulting in the substitution of isoleucine with threonine at position 232 (140), excluding the receptor from lipid rafts. This prevents interaction of FCGR2B with ITAM containing receptors such as the activating FCGR and the BCR (141, 142). Mφs from individuals homozygous for $FCGR2B^{T232}$ showed a stronger phagocytic capacity of IgG-IC while the B cells of these individuals showed reduced FCGR2B-mediated inhibition of BCR-triggered proliferation (142).

GWAS analyses have shown an association between *rs1050501* and SLE (140, 143–147). Three meta-analyses confirmed these associations (147–149). The $FCGR2B^{T232}$ homozygosity is associated with an odds ratio of 1.73, one of the strongest associations in SLE (147). Association of *rs1050501* with Rheumatoid Arthritis (RA) has been reported for a Taiwanese cohort (150).

The frequency of homozygosity of the $FCGR2B^{T232}$ allele is only 1% in Caucasians and in contrast 5–11% in African and South-East Asian populations (151). This might be one of the explanations for the ethnic differences in SLE susceptibility. Malaria is endemic in Africa and South-East Asia. An association was found between decreased susceptibility for severe malaria and homozygosity for the $FCGR2B^{T232}$ allele (135). So, increased protection against malaria by down-regulation of FCGR2B expression goes along with increased risk to develop SLE.

A significant but weak association has been observed between *SLAMF* and susceptibility to SLE. The weakness of the association might be explained by the limited size of the cohorts studied (152). An association study of UK and Canadian families with SLE has revealed multiple polymorphisms in several *SLAMF* genes (153). However, the strongest association with a non-synonymous SNP could not be replicated in independent Japanese and European cohorts of SLE patients (154, 155). Instead, another SNP was significantly associated with the susceptibility to SLE in another Japanese cohort (156). One large-scale case-control association study showed an association of two SNPs with increased susceptibility to RA, in two independent Japanese cohorts (155). In conclusion, these observations indicate that also in human's polymorphisms of *SLAMF* contribute to the susceptibility to autoimmune disease.

Overall, a model emerges from both studies with C57BL/6 *Sle* congenic mouse strains and human SLE (157), in which disease susceptibility arises through the co-expression of multiple genetic variants that have weak individual effects (152, 158). According to the "threshold liability" model, the severity of the autoimmune phenotype increases with the increasing number of autoimmunity associated allelic variants of autoimmune susceptibility genes in the genome. However, epistatic interactions might result in a more complex non-additive inheritance of the autoimmune phenotype (**Figure 2**). According to this "multiplicative model" the interactions of all susceptibility and suppressor alleles in the genome determine the susceptibility for autoimmune diseases of an individual (159). Importantly this means that the contribution of an individual gene to the autoimmune phenotype can vary depending on the presence of other susceptibility and suppressor genes in the genome (the genomic context). This might explain the puzzling and contradictory results with the $Fc\gamma RIIb_{NZW}$ and $Fc\gamma RIIB_{NZB}$ haplotypes. To uncover the polygenic effects associated with a complex disease such as SLE not a single gene association approach but gene set analysis (GSA) is required (160). However, a reverse genetic approach might offer the opportunity to reconstruct an autoimmune phenotype by modifying a combination of a limited number of candidate genes in a well-defined genetic background.

REVERSE GENETICS

So far three *FcγRIIb* KO mouse models have been published. The first published KO was generated by gene targeting in 129 derived ES cells (161) and subsequently backcrossed into the C57BL/6 background, here called $Fc\gamma RIIb_{129}^{-/-}$ mouse. This mouse on a not well-defined mixed genetic background was during 15 years (between 1996 and 2011) the only *FcγRIIb* KO model available and has been extensively used resulting in an overwhelming amount of literature concerning the role of FcγRIIb in immune tolerance. Subsequently, independently, in two different laboratories *FcγRIIb* KO mice were generated by gene targeting in C57BL/6 derived ES cells, here called $Fc\gamma RIIb_{B6}^{-/-}$ mice (109, 110). The published data regarding ANA titers of one of these mouse strains are inconsistent (162, 163) as are the autoimmune phenotypes of both C57BL/6 strains (109, 110). Moreover, it is still under debate to what extent the autoimmune phenotypes of the $Fc\gamma RIIb_{B6}^{-/-}$ mice differ from the autoimmune phenotype of the $Fc\gamma RIIb_{129}^{-/-}$ mice. Therefore, we discuss in chronological order these different models.

The *FcγRIIb* KO Mouse on Mixed 129/C57BL/6 Background

The $Fc\gamma RIIb_{129}^{-/-}$ mouse develops elevated immunoglobulin levels in response to both T cell-dependent and T cell-independent Ags (161), have more plasma cells (10), and show an enhanced passive cutaneous anaphylaxis compared to WT controls (161). They develop arthritis (164) and Good pasture's syndrome-like disease (165) upon immunization with bovine collagen type II and type IV, respectively when backcrossed into the non-permissive ($H\text{-}2^b$ haplotype) C57BL/6 background. When backcrossed more than 7 generations into C57BL/6, but not BALB/c background, the $Fc\gamma RIIb_{129}^{-/-}$ mice started to develop spontaneously with high penetrance lupus-like disease. This autoimmune disease is characterized by gender bias, splenomegaly, increase of the proportion of different subsets of activated lymphocytes with age, high titers of ANA, IC-mediated GN and vasculitis in different organs resulting in proteinuria and premature death (112) very similar to the phenotype of the NZM2410 mouse we discussed earlier. This is surprising because, as we have seen, genetic studies revealed that lupus susceptibility is a multigenic phenotype. Monogenic autoimmune diseases are rare (158). However, the strong autoimmune phenotype of the $Fc\gamma RIIb_{129}^{-/-}$ mouse cannot be attributed exclusively to the deletion of the *FcgRIIb* alleles. This mouse has been generated by gene targeting in 129 derived ES cells and subsequently

backcrossed into C57BL/6 background. Such a mouse is, even after 10–12 generations, not fully C57BL/6 but congenic for the 129 derived flanking regions of the targeted allele, containing still hundreds of genes of 129 origin (**Figure 1**). The 129 genome contains more than 1,000 non-synonymous mutations compared to the C57BL/6 genome (166). This is only one part of the problem. Epistasis between 129 derived loci and the C57BL/6 genome also occurs. It has been shown that mice without targeted alleles but congenic for the 129 derived distal-region of Chr1 (*Sle16*), a lupus-associated region including the autoimmune-prone haplotype 2 of the *Slamf* genes and the haplotype I of the *FcγRIIb* gene, develop a similar autoimmune phenotype as C57BL/6 *Sle1* congenic mice (111). That might explain why several mouse strains generated by targeting genes in the proximity of the *Slamf* locus in 129 derived ES cells, and backcrossed into C57BL/6 background, develop autoimmunity.

Strikingly, the $FcγRIIb_{129}^{-/-}$ mouse backcrossed more than seven generations into C57BL/6 background develops ANA with similar selective reactivity to H2A/H2B/DNA sub-nucleosomes as C57BL/6 *Sle1* congenic mice, however, with earlier onset, stronger penetrance, and higher titers. Irradiated $Rag^{-/-}$ C57BL/6 or $IgH^{-/-}$ C57BL/6 mice adoptively transferred with bone marrow from $FcγRIIb_{129}^{-/-}$ mice backcrossed more than seven generations into C57BL/6 background developed anti-chromatin antibodies and proteinuria, indicating that the disease is fully transferable, dependent on B cells. Myeloid $FcγRIIb^{-/-}$ cells are not required (112). This is in keeping with experiments, mentioned earlier, that show that the autoimmune phenotype of C57BL/6 *Sle1* congenic mice is completely reconstituted in C57BL/6 irradiated mice that received bone marrow from C57BL/6 *Sle1* congenic mice but not by the reciprocal reconstitution. This demonstrates that *Sle1* is functionally expressed in B cells (101) although impaired FcγRIIb expression seems to play a minor role in that model (113, 117). Taken together these data all point in the same direction: the strong lupus-like phenotype of the $FcγRIIb_{129}^{-/-}$ mice backcrossed more than seven generations into C57BL/6 background is caused by epistatic interaction between the $Slamf_{129}$ locus, the C57BL/6 genome, and $FcγRIIb^{-/-}$ (**Figure 2**), similar to the epistatic interactions between $FcγRIIb_{NZB}$ (haplotype I), $Slamf_{NZB}$ (haplotype 2) and the C57BL/6 genome in C57BL/6 *Nba2* congenic mice (114). As a consequence, the $FcγRIIb_{129}^{-/-}$ mouse suffers from the confounding effect that the $FcγRIIb_{129}$KO alleles are closely linked to the $Slamf_{129}$ locus associated with autoimmunity. This means that in most experimental conditions, no distinction can be made between $FcγRIIb^{-/-}$ and $Slamf_{129}$ mediated effects in these mice.

Ig gene analysis of ANA suggests that ANA develop in GCs (167–172). Therefore, analysis of the loss of tolerance in $FcγRIIb_{129}^{-/-}$ mice focused on GC (173). The role of FcγRIIb as an immune tolerance checkpoint has been studied in a transgenic mouse model in which the variable heavy chain (V_H) *3H9H-56R*, derived from a dsDNA specific hybridoma, or its variant $56RV_H$, with higher affinity binding to dsDNA, were inserted in the *Igh* locus (IgM^a allele) (174). Receptor editing, based on the use of specific light chains that abrogates the dsDNA binding, is the main mechanism to maintain tolerance in these mice (175–177). The Ab selection process was compared between WT C57BL/6 and $FcγRIIb_{129}^{-/-}$ mice carrying the V_H transgenes (178). C57BL/6 mice expressing the high-affinity *56R* allele (B6.*56R*) developed low but significant anti-DNA titers, indicating that tolerance was broken, whereas C57BL/6 mice with the low-affinity *3H9* allele (B6.*3H9*) did not. Tolerance was also maintained in $FcγRIIb_{129}^{-/-}$ mice carrying the low-affinity *3H9* allele ($FcγRIIb_{129}^{-/-}$.*3H9*). The development of IgM-positive autoreactive B cells was similar in $FcγRIIb_{129}^{-/-}$ mice carrying the high-affinity *56R* allele ($FcγRIIb_{129}^{-/-}$.*56R*) and B6.*56R* mice. Moreover, $FcγRIIb_{129}^{-/-}$.*3H9* mice and $FcγRIIb_{129}^{-/-}$.*56R* mice did not show differences in the populations of activated and GC B cells or T cells compared to B6.*3H9* and B6.*56R* control mice. However, $FcγRIIb_{129}^{-/-}$.*56R* mice developed higher IgG anti-DNA titers compared to B6.*56R* mice. Taking together these observations suggest that the function of FcγRIIb in B6.*56R* mice is limiting the production of serum IgG anti-dsDNA. Analysis of hybridomas derived from these different mouse strains showed that a much higher percentage of hybridomas from $FcγRIIb_{129}^{-/-}$.*56R* mice secreted IgG antibodies compared to the hybridomas from B6.*56R* mice. Moreover, $FcγRIIb_{129}^{-/-}$.*56R* mice had a higher percentage of splenocytes with a plasma cell phenotype compared to B6.*56R* mice. The cross of the $FcγRIIb_{129}^{-/-}$ mice with autoimmune B cell receptor transgenic mice most likely bypasses the involvement of $Slamf_{129}$ (which is mainly responsible for the spontaneous development of autoreactive B cells in a C57BL/6 $Slamf_{129}$ congenic strain, as we will see later). So, in this case, the phenotype of the $FcγRIIb_{129}^{-/-}$.*56R* mouse can be completely attributed to the absence of FcγRIIb. From these results, it was concluded that the main function of FcγRIIb in the GC reaction is to control, as one of the latest checkpoints, the development of autoreactive IgG-secreting plasma cells and that most likely FcγRIIb deficiency modifies autoimmunity rather than initiates loss of tolerance (178). This was confirmed independently, in an experimental model with two V_H chain knockin strains, HKI65 and HKIR, with specificity for the hapten arsonate and a weak and strong specificity for DNA respectively (179). No indications for a role of FcγRIIb in primary or GC tolerance checkpoints were found. Only an increased number of plasma cells was detected in mice that received C57BL/6 *HKIR*/$FcγRIIb_{129}^{-/-}$ B cells. FcγRIIb seems to prevent autoimmunity by suppressing the production of autoreactive IgG from B cells that escaped negative selection in GC and enter the AFC pathway (179). This is also in agreement with observations in C57BL/6 $FcγRIIB_{NZW}$ congenic mice mentioned earlier (108). However, more recently it has been shown that the number of spontaneous (Spt) GC B cells is increased in 6–7 months old $FcγRIIb^{-/-}$ mice on a pure C57BL/6 background, suggesting that FcγRIIb deficiency dysregulates the Spt-GC B cell response [(163); **Table 3**] as will be discussed later.

The view that FcγRIIb acts as a suppressor of autoimmunity caused by other loci is supported by the observed synergism between $FcγRIIb^{-/-}$ and several autoimmune susceptibility loci. Just like the *Sle1* locus (100), $FcγRIIb^{-/-}$ interacts synergistically

with the autoimmune susceptibility *Yaa* locus from BXSB autoimmune-prone mice, containing the *Tlr7* gene translocated from the X chromosome to the Y chromosome, resulting in strong acceleration of lupus-like disease in $Yaa^{+}Fc\gamma RIIb^{-/-}_{129}$ male mice (113) (**Figure 2**). MRL/$Fas^{lpr/lpr}$ mice develop lupus-like disease whereas C57BL/6 $Fas^{lpr/lpr}$ mice do not, likely due to suppressor activity of the C57BL/6 genome. However, C57BL/6 $Fas^{lpr/lpr}Fc\gamma RIIb^{-/-}_{129}$ mice develop systemic autoimmune disease (180). This is consistent with the presence of the haplotype I allelic variant of *FcγRIIb* in MRL mice with an impaired expression on B cell subsets. Mice deficient for both, deoxyribonuclease 1 like 3 (DNASE1L3) and FcγRIIb exhibit at the age of 10 weeks an IgG anti-dsDNA production higher than in 9 months old (NZBxNZW)F1 mice (181). The presence of either the *Yaa* locus or homozygosity for the Fas^{lpr} or *Dnase1l3* KO alleles is most likely sufficient to break tolerance. However, FcγRIIb prevents strong autoimmunity by suppressing the production of autoreactive IgG from B cells that have escaped negative selection and enter the AFC pathway. Because $Fc\gamma RIIb^{-/-}_{129}$ mice were used in the crosses mentioned a role for $Slamf_{129}$ cannot be excluded in these models as indicated by the much milder phenotype of the Yaa^{+} $Fc\gamma RIIb^{-/-}_{B6}$ mouse on pure C57BL/6 background discussed later (109) compared to the severe lupus phenotype of the $Yaa^{+}Fc\gamma RIIb^{-/-}_{129}$ mouse (**Figure 2**). Nevertheless, these observations underscore the crucial role of FcγRIIb in the protection against the development of spontaneous autoimmunity determined by other autoimmune susceptibility loci.

Because of allelic exclusion, Ig transgenic mice do not have a normal B cell repertoire. Therefore, the development of self-reactive GC B cells and plasma cells was studied in $Fc\gamma RIIb^{-/-}_{129}$ mice by large scale Ig cloning from single isolated B cells to determine how loss of FcγRIIb influences the frequency at which autoreactive ANA-expressing B cells participate in GC reactions and develop in plasma cells under physiological conditions (173). In comparison with WT controls the following was observed in $Fc\gamma RIIb^{-/-}_{129}$ mice: (a) No skewing of *Ig* gene repertoire but enrichment for IgGs with highly positively charged IgH CDR3s which is associated with antibody autoreactivity; (b) lower numbers of somatic mutation; (c) increased numbers of polyreactive IgG^{+} GC B cells and bone marrow plasma cells and (d) enrichment of nucleosome-reactive GC B cells and plasma cells. The overall frequency of ANAs was high in GC B cells but not in plasma cells. These results demonstrate that in $Fc\gamma RIIb^{-/-}_{129}$ mice IgG autoantibodies including ANAs are expressed by GC B cells and that somatic mutations contribute to the generation of high-affinity IgG antibodies suggesting that the $Fc\gamma RIIb^{-/-}$/$Slamf_{129}$ combination plays an important role in the regulation of autoreactive IgG^{+} B cells which develop from non-self-reactive or low-self-reactive precursors by affinity maturation (173). It would be of great interest to repeat this analysis in $Fc\gamma RIIb^{-/-}_{B6}$ mice on pure C57BL6 background and C57BL/6 $Slamf_{129}$ congenic mice to define the individual contribution of the $Slamf_{129}$ locus and the *FcγRIIb* KO alleles in the loss of immune tolerance in the C57BL/6 background. Interestingly the frequency of high-affinity autoreactive IgG^{+} plasma cells was relatively low, given the high frequency of autoreactive IgG^{+} GC B cells. This can be explained by the existence of a tolerance checkpoint before GC B cells differentiate into spleen or bone marrow plasma cells, downstream of FcγRIIb and Slamf (173).

Complementation of the mutant phenotype of an organism by expression of a transduced WT gene is considered as the ultimate proof that the mutated gene is the cause of the phenotype. Irradiated autoimmune-prone BXSB, NZM2410, and $Fc\gamma RIIb^{-/-}_{129}$ mice transplanted with autologous bone marrow transduced with a viral vector expressing FcγRIIb showed reduced autoantibody levels and as a consequence much milder disease symptoms compared to mice that received autologous bone marrow transduced with an empty vector (182). These results were confirmed by using a transgenic mouse with a stable 2-fold B cell-specific overexpression of FcγRIIb (183). These mice hardly developed a lupus-like disease when backcrossed into autoimmune-prone MRL/$Fas^{lpr/lpr}$ background. The underlying mechanism of these strong effects of overexpression of FcγRIIb is not known. These experiments mainly demonstrate that overexpression of FcγRIIb on B cells inactivates these cells resulting in a strong decrease in autoantibody production. Although they confirm a role of FcγRIIb in autoimmune disease they don't answer the intriguing question whether FcγRIIb deficiency is a modifier of autoimmunity rather than a primary initiator of the loss of tolerance.

FcγRIIb KO on a Pure C57BL/6 Background

To avoid the confounding effect of 129 derived flanking sequences (*Sle16*), independently, in two different laboratories $Fc\gamma RIIb^{-/-}$ mice were generated by gene targeting in C57BL/6 ES cells. To distinguish between these two models, one is called here $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ (109) and the other $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ (110). $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mice exhibit a hyperactive phenotype in the effector phase, although somewhat milder than $Fc\gamma RIIb^{-/-}_{129}$ mice, suggesting a contribution of *Sle16* to the phenotype of the $Fc\gamma RIIb^{-/-}_{129}$ mouse in the effector phase (109). Both KO mice develop very mild lupus-like disease (**Table 2**). Total IgG ANA was not significantly increased in 10 months old female $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mice compared to C57BL/6 mice although serum of 5% of these mice showed some total IgG anti-dsDNA and anti-ssDNA antibody titers just above (C57BL/6) baseline. In contrast, in 40% of 10 months old $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mice total IgG anti-nuclear Abs was significantly increased compared to C57BL/6 mice (110). But only five percent of $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mice showed premature death whereas mortality was not increased in $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mice although proteinuria and kidney pathology were significantly higher in these mice compared to C57BL/6 mice. The kidney phenotype in the absence of detectable ANA in $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mice points to a protective role of FcγRIIb in the kidney, in the efferent phase, as has also been shown in a model

TABLE 2 | Disease phenotypes of $Fc\gamma RIIb^{-/-}_{B6}$, C57BL/6 $Fc\gamma RIIB^{-/-}_{129}$ $Slamf_{B6}$ congenic, C57BL/6 $Slamf_{129}$ congenic and the original $Fc\gamma RIIb^{-/-}_{129}$ mice compared to WT C57BL/6 control mice at the age of 6–8 months.

Phenotype \ Mouse	$^{Le}Fc\gamma RIIb^{-/-}_{B6}$ [c]	*C57BL6 $Fc\gamma RIIb^{-/-}_{129}$ $Slamf_{B6}$* Congenic[a]	$^{NY}Fc\gamma RIIb^{-/-}_{B6}$ [b,d]	*C57BL/6 $Slamf_{129}$* Congenic[a,b]	$Fc\gamma RIIb^{-/-}_{129}$ [a,b,c,d]
Increased IgM	n.d.	–[a]	n.d.	–[a]	+[a]
Increased IgG[a]	n.d.	+ (♀)[a]	n.d.	–[a]	+ (♀ ♂)[a]
α-DNA	+ (♀) Total IgG Incidence 5%[c]	+ (♀) IgG2c[a]	++ (♀) IgG2c[b] Total IgG[d]	+++ IgG2c/2b[b] (♀) IgG2c[a]	+++++++ (♀) IgG2c[a,b] IgG2b[b] Total IgG[a,d]
α-histone	– (♀) Total IgG[c]	n.d.	++ (♀) IgG2c[b]	+++ IgG2c/2b[b]	+++++++ IgG2c/2b[b] Total IgG[c]
α-nuclear	+ (♀) Total IgG[c]	+ (♀) IgG2c[a]	++ (♀) IgG2c[b] Total IgG Incidence 40%[d]	+++ IgG2c[a,b] IgG2b[b]	+++++++ (♀) IgG2c[a,b] IgG2b[b] Total IgG[a,d]
Kidney pathology	+ (♀)[c]	+ (♀)[a]	++[b]	–[a,b]	+++++[a,b,c]
IgG-IC deposition in glomeruli	+ (♀)[c]	+ (♀)[a]	++ (♀)[b]	+ (♀)[b] – (♀)[a]	++++ (♀ ♂)[a,b,c]
C3 deposition	+[c]	n.d.	–[b]	+[b]	+++++[b,c]
Spleen	Slightly enlarged (♀)[c]	Slightly enlarged (♀)[a]	n.d.	Slightly enlarged (♀)[a]	Splenomegaly[a,b,c]
Spt-GC formation	n.d.	Normal (♀ ♂)[a]	Augmented + (♀)[b]	Augmented ++ (♀)[a,b]	Augmented +++ (♀)[a,b]
% GC B cells of $CD19^+$ splenic B cells	n.d.	No increase (♀)[a]	Increase +[b]	Increase ++ (♀)[a]	Increase +++ (♀)[a]
Absolute numbers of splenic GC B cells	n.d.	No increase (♀)[a]	n.d.	Increase + (♀)[a]	Increase ++ (♀)[a]
Increased Mortality	–[c]	–[a]	+ 5%[d]	–[a,b]	Varies from 0%[a] (and 22%[c]) to 60%[d]

[a] *Kanari et al. (98).*
[b] *Soni et al. (163).*
[c] *Boross et al. (109).*
[d] *Li et al. (110).*
n.d., not determined.

of antibody-induced nephrotoxic nephritis (NTN) that will be discussed later (89).

The production of autoantibodies by C57BL/6 mice in the absence of FcγRIIb suggests that FcγRIIb deficiency, besides modifying autoimmunity caused by other autoimmune susceptibility loci (e.g., $Slamf_{129}$, *Yaa*), as discussed earlier, can result in loss of tolerance in the GC. However, it is tempting to speculate that the low titers of autoantibodies, that develop with low penetrance in *FcγRIIb* KO mice on a pure C57BL/6 background, reflect the natural occurring autoreactive B cells in the GC of a WT C57BL/6 mouse, as described earlier, that are prevented to enter the AFC pathway in the presence of FcγRIIb (178). There are indications that C57BL/6 mice are more autoimmune prone than BALB/c mice. For example, B cell receptor editing as a mechanism to maintain B cell tolerance is less effective in these mice compared to BALB/c mice (178).

The $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mouse seems to exhibit a stronger disease phenotype than the $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mouse (**Table 2**). There are several explanations for this discrepancy:

a. The strains are generated with different ES cell lines. There might be relevant genomic differences between the C57BL/6 derived ES cell lines used. This question can be answered by sequencing the *FcγRIIb* flanking genomic regions in both mouse strains.
b. The mice have been backcrossed several generations into different C57BL/6 mouse strains. There are substantial genetic variations between the different C57BL/6 strains used in different laboratories (184).
c. Environmental factors (immune status, microbiome) play a role. The incidence of lethal disease in $Fc\gamma RIIb^{-/-}_{129}$ mice varies between different laboratories from 0% to more than 60% (98, 109, 112, 173).
d. Differences in the methods used to measure ANA. In the $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mouse ANA have been measured only by ELISA of total IgG (109), whereas in the $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mouse IgG2a and IgG2b have been measured combined with Hep-2 cell staining (163). However, a significant increase in total IgG anti-nuclear Abs compared to C57BL/6 has also been reported with the $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mouse (110).

TABLE 3 | Characteristics of GC B and T cells in $^{NY}Fc\gamma RIIb^{-/-}_{B6}$, C57BL/6 $Slamf_{129}$ congenic, and the original $Fc\gamma RIIb^{-/-}_{129}$ mice compared with WT C57BL/6 control mice.

Phenotype \ Mouse strain	$Fc\gamma RIIb^{-/-}_{129}$	C57BL/6 $Slamf_{129}$ congenic	$^{NY}Fc\gamma RIIb^{-/-}_{B6}$
Increase in frequency of $B220^{+}PNA^{hi}$ $CD95^{hi}$ Spt-GC B cells	++++	++	+
Increase in Splenic GC size	+++	++	+
Increase in frequency of $CD4^{+}CXCR5^{hi}PD\text{-}1^{hi}$ GC T_{FH} cells	+++	+	–
Increase in frequency of $CD4^{+}CXCR5^{int}PD\text{-}1^{int}$ T_{FH} cells	+++	+	–
Increase in $CD4^{+}GL7^{+}$ GC T_{FH} cells	++	+	–
IL-21 expression in GC T_{FH} cells	++++	++	–
PD-1 expression in GC T_{FH} cells	++++	++	++
ICOS expression in GC T_{FH} cells	++	–	–
Increase in frequency of GC B cells upon antigenic stimulation	n.d.	+	–
Increase in frequency of GC T_{FH} cells upon antigenic stimulation	n.d.	+	–
MHC class II upregulation on GC B cells upon antigenic stimulation	n.d.	+	–
Decrease of caspase activity in $DAPI^{neg}B220^{+}Fas^{hi}PNA^{hi}$ GC B cells	++	+/–	+/–

n.d., not determined (163).

The Individual Contribution of FcγRIIb Deficiency and $Slamf_{129}$ to the Phenotype of the FcγRIIb KO Mouse on Mixed 129/C57BL/6 Background

Independently, in two different laboratories congenic C57BL6 $Slamf_{129}$ mice have been generated. One was generated by intensive backcrossing of the original $Fc\gamma RIIb^{-/-}_{129}$ mouse (161) into C57BL/6 background and selection for offspring in which the *Slamf* locus and the $Fc\gamma RIIb$ KO allele had been segregated (98) resulting in two congenic strains called here as C57BL/6 $Slamf_{129}$ congenic and C57BL/6 $Fc\gamma RIIb^{-/-}_{129}$ $Slamf_{B6}$ congenic, respectively. The other C57BL6 $Slamf_{129}$ congenic mice were generated by a marker-assisted speed congenic approach (163) (**Figure 1**).

The development of autoimmunity was compared between C57BL/6 $Fc\gamma RIIb^{-/-}_{129}$ $Slamf_{B6}$ congenic, C57BL/6 $Slamf_{129}$ congenic and the original $Fc\gamma RIIb^{-/-}_{129}$ mice (98) or between C57BL/6 $Slamf_{129}$ congenic, $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ and the original $Fc\gamma RIIb^{-/-}_{129}$ mice (163). Both C57BL/6 $Fc\gamma RIIb^{-/-}_{129}$ $Slamf_{B6}$ congenic and C57BL/6 $Slamf_{129}$ congenic mice developed very mild disease symptoms whereas the original $Fc\gamma RIIb^{-/-}_{129}$ mice developed severe disease compared to WT C57BL/6 mice. Importantly, the phenotype of the C57BL/6 $Fc\gamma RIIb^{-/-}_{129}$ $Slamf_{B6}$ congenic mouse strain confirmed mainly the phenotype of the $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mouse [(98); **Table 2**] showing very low ANA titers and little kidney pathology compared to $Fc\gamma RIIb^{-/-}_{129}$ mice.

The development of Spt-GC B cell and T_{FH} responses in C57BL/6 $Slamf_{129}$ congenic, $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ and $Fc\gamma RIIb^{-/-}_{129}$ mice were carefully compared [(163); **Table 3**]. C57Bl/6 $Slamf_{129}$ congenic mice had significantly more GC B cells and T_{FH} and GC T_{FH} cells 12 days after immunization with OVA compared to WT C57BL/6 mice. B cells and DCs from $Slamf_{129}$ congenic mice exhibited stronger antigen presentation in *in vitro* assays compared to B cells and DCs from WT C57BL/6 mice. By using a variety of *in vivo* and *in vitro* assays with naïve B cells it was found that B cell-intrinsic deficiency of FcγRIIb and expression of $Slamf_{129}$ has no effect on proliferation but promotes differentiation of naïve B cells into GC B cells as indicated by increased expression of Aicda and GL-7. The percentage of apoptotic GC B cells was significantly lower in $Fc\gamma RIIb^{-/-}_{129}$ mice compared to WT C57BL/6 mice whereas in C57BL/6 $Slamf_{129}$ congenic and $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mice this decrease was not significant. This suggests that FcγRIIb deficiency and $Slamf_{129}$ act synergistically to increase the survival of GC B cells in $Fc\gamma RIIb^{-/-}_{129}$ mice. Naïve and activated B cells from $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ and to a lower extent from C57BL/6 $Slamf_{129}$ congenic mice showed an enhanced metabolic capacity compared to B cells from C57BL/6 mice. This enhancement was stronger in $Fc\gamma RIIb^{-/-}_{129}$ mice.

Taken together these observations suggest that $Slamf_{129}$ plays a predominant, and FcγRIIb deficiency a modest role in modulating the Spt-GC B cell and T_{FH} responses. Some of their functions are synergistic others mutually exclusive. GC T_{FH} cell responses are mainly affected by $Slamf_{129}$ [(163); **Table 3**]. By using the experimental model of the V_H chain knockin strain HKIR mentioned earlier (179) it was demonstrated that B cell-specific expression of $Slamf_{129}$ is necessary for the autoreactive B cells to expand in the GC confirming previous observations in C57BL/6 *Sle1* congenic mice (129).

The increased Spt-GC responses in $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ and C57BL/6 $Slamf_{129}$ congenic mice were associated with the production of autoantibodies. However, the titers were much lower than in $Fc\gamma RIIb^{-/-}_{129}$ mice which had also the strongest increase in Spt-GC responses. C57BL/6 $Slamf_{129}$ congenic mice developed higher ANA titers than $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mice, staining both cytoplasm and nucleus of Hep-2 cells, whereas sera from $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mice show only cytoplasmic staining patterns (163) confirming previous results with the C57BL/6 $Fc\gamma RIIb^{-/-}_{129}$ $Slamf_{B6}$ congenic mouse strain (98). IgG2b and IgG2c ANA

were significantly increased in C57BL/6 $Slamf_{129}$ congenic mice whereas only IgG2c ANA were significantly increased in $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ mice. With an autoantigen array, it was shown that $Fc\gamma RIIb_{129}^{-/-}$ mice develop high titers of IgG antibodies against a large variety of autoantigens. Several of these antibodies were also present in the serum of $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ mice but their titers were much lower than in $Fc\gamma RIIb_{129}^{-/-}$ mice (163). Unfortunately, sera from C57BL/6 $Slamf_{129}$ congenic mice were not tested in the autoantigen array.

Kidney pathology was absent (98) or very mild, with higher complement deposition than $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ mice (163), in C57BL/6 $Slamf_{129}$ congenic mice, mild in $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ or C57BL/6 $Fc\gamma RIIb_{129}^{-/-}$ $Slamf_{B6}$ congenic mice with higher IgG deposition than in C57BL/6 $Slamf_{129}$ congenic mice, and severe, with highest C3 and IgG deposition compared to the other genotypes, in $Fc\gamma RIIb_{129}^{-/-}$ mice (98, 163). In conclusion, the deficiency of FcγRIIb together with the presence of $Slamf_{129}$ results in a phenotype of the $Fc\gamma RIIb_{129}^{-/-}$ mouse with increased Spt-GC B cell responses characterized by an increase of the following parameters: metabolic activity in B cells, differentiation of B cells into a GC B cell phenotype and GC B cell survival. This is associated with loss of immune tolerance resulting in ANA production and the development of severe lupus-like disease (163). However, the underlying cellular and molecular mechanisms of these associations are not well-understood and the subject of speculation and debate with respect to the role of FcγRIIb in GC (185). This can be illustrated with the surprising observation in the $Fc\gamma RIIb_{NZB}$ KI mouse model mentioned earlier, in which FcγRIIb failed to be upregulated on activated and GC B cells resulting in enhanced early GC responses (11). Upon immunization, these KI mice showed an early and sustained increased affinity maturation of Ag-specific GC B cells. Previous models suggest that low expression of FcγRIIb reduces the BCR activation threshold resulting in less affinity maturation. However, an alternative explanation might be that low FcγRIIb expression increases the survival of bystander Ag non-specific GC B cells and, as a consequence, increases competition for T_{FH} help between Ag-specific and non-antigen specific B cells, resulting in increased affinity maturation (11).

Cell-Type-Specific *FcγRIIb* KO Mouse Models

To determine on what B cell subset(s) and on what myeloid cells FcγRIIb might be involved in a checkpoint for immune tolerance, cell-type-specific $Fc\gamma RIIb^{-/-}$ mice were generated, independently, in two different laboratories. Both the $^{Le}Fc\gamma RIIb_{B6}^{-/-}$ and $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ mouse models, on a pure C57BL/6 background, were originally generated as floxed $Fc\gamma RIIb$ mice ($Fc\gamma RIIb_{B6}^{fl/fl}$) and subsequently crossed with a Cre deleter transgenic mouse to generate the germline $Fc\gamma RIIb_{B6}^{-/-}$ mice discussed earlier. In addition, the $Fc\gamma RIIb_{B6}^{fl/fl}$ mice were also crossed with a variety of cell type-specific Cre transgenic mice (**Table 4**) to generate cell-type-specific $Fc\gamma RIIb_{B6}^{-/-}$ strains that were analyzed in the following models of diseases for which germline $Fc\gamma RIIb$ KOs are highly susceptible: (a) the induced autoimmune diseases CIA, both on permissive (immunization with chicken collagen type II) and non-permissive (immunization with bovine collagen type II) background and (b) anti-glomerular basement membrane antibody (anti-GBM) disease, (c) the spontaneous autoimmune disease lupus-like disease and (d) the non-autoimmune disease antibody-induced NTN.

Deletion of $Fc\gamma RIIb$ in all B cells of the $^{Le}Fc\gamma RIIb_{B6}^{fl/fl}$ mouse by *CD19Cre* did not increase the susceptibility of this mouse for any of the mentioned disease models. Moreover, deletion of $Fc\gamma RIIb$ on a subset of monocytes (*LysMCre*) had no effect on susceptibility for anti-GBM disease. Therefore, it was concluded that FcγRIIb deficiency on B cells or a subset of myeloid cells alone is not sufficient to increase susceptibility to anti-GBM (186). Only pan-myeloid deletion (*cEBPαCre*) of FcγRIIb increased the susceptibility of $^{Le}Fc\gamma RIIb_{B6}^{fl/fl}$ mice for CIA on the permissive background (187) and for the non-autoimmune disease NTN (89). These results suggest that for the protection against induced auto-Ab driven diseases, such as CIA, the role of FcγRIIb on B cells, as a checkpoint for immune tolerance, is less important than its role on myeloid effector cells, controlling downstream antibody effector mechanisms (187). However, it cannot be excluded that in the CIA model FcγRIIb on myeloid cells also plays a role in controlling the afferent phase of the disease, as was recently shown in Yaa^{+} $^{Le}Fc\gamma RIIb_{B6}^{-/-}$ mice that will be discussed later (188).

In contrast to the results with $^{Le}Fc\gamma IIb_{B6}^{fl/fl}$ mice, deletion of FcγRIIb in all B cells (*Mb1Cre*) or in GC and post GC B cells (*Cg1Cre*) in $^{NY}Fc\gamma RIIb_{B6}^{fl/fl}$ mice resulted in increased susceptibility for CIA on the non-permissive background and permissive background, respectively. Moreover, susceptibility to CIA was also increased in DC-specific *CD11cCre*/$^{NY}Fc\gamma RIIb_{B6}^{fl/fl}$ mice indicating that FcγRIIb is involved in distinct immune tolerance controlling mechanisms (110). The reason for the discrepancy between the phenotypes of the B cell- and DC-specific $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ and $^{Le}Fc\gamma IIb_{B6}^{-/-}$ mice is not known but, given the weak phenotype of the germline $^{Le}Fc\gamma RIIb_{B6}^{-/-}$ mouse, most likely the phenotype of a single cell-type-specific $^{Le}Fc\gamma RIIb_{B6}^{-/-}$ mouse is too weak to be detected with a small cohort of mice. Another partial explanation might be that the B-cell-specific Cre lines used are different. In addition, GC and post GC B cell (*Cg1Cre*) specific $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ mice developed spontaneously ANA, similar to ANA in germline $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ mice, whereas a deficiency in other cell types has no effect. This confirms previous results with transplantation of bone marrow from $Fc\gamma RIIb_{129}^{-/-}$ mice that the role of FcγRIIb in the spontaneous development of ANA is B cell-specific (112) and suggests that FcγRIIb on GC or post GC B cells is a checkpoint for the maintenance of immune tolerance (110) (**Table 4**).

Upon immunization with the NP-CGG model antigen $^{NY}Fc\gamma RIIb_{B6}^{-/-}$ and *Mb1Cre*/$^{NY}Fc\gamma RIIb_{B6}^{fl/fl}$ mice developed similar increased primary IgG NP-specific Ab responses compared to $^{NY}Fc\gamma RIIb_{B6}^{fl/fl}$ mice and all other cell type-specific

TABLE 4 | Disease susceptibility of cell-type-specific *FcγRIIb* KO mice.

Disease \ Mouse strain	***CD19Cre:* *All B cells*[a,b,c]**	***LysMCre:* *Subset monocytes*[a,d]**	***cEBPαCre:* *pan-myeloid*[b,c]**	***CD11cCre:* *DCs*[c,d]**	***Mb1Cre:* *All B cells*[d]**	***Cg1Cre:* *GC and post GC B cells*[d]**
Non-permissive bCIA[c,d]	No increase	No increase $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	n.d.	Increase $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	Increase $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	No increase $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]
Permissive cCIA[c,d]	No increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [c]	n.d.	Increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [c]	No increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [c]	n.d.	Increase similar to $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ [d]
KRN arthritis[d]	n.d.	Increase $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	n.d.	n.d.	n.d.	n.d.
Anti-GBM disease[a]	No increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [a]	No increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [a]	n.d.	n.d.	n.d.	n.d.
Lupus-like disease[d]	n.d.	No ANA $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	n.d.	No ANA $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	No ANA $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	ANA similar to $^{NY}Fc\gamma RIIb^{-/-}_{B6}$ [d]
NTN[b]	No increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [b]	n.d.	Increase $^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ [b]	n.d.	n.d.	n.d.
Immunization[d]	n.d.	No increase in IgG response $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	n.d.	No increase in IgG response $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	Increased primary/secondary IgG response $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]	Increased secondary IgG response $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ [d]

Germline FcγRIIb KO mice showed increased susceptibility to all diseases listed in the table compared with C57BL/6 mice.
[a] *Sharp et al. (186).*
[b] *Sharp et al. (89).*
[c] *Yilmaz-Elis et al. (187).*
[d] *Li et al. (110).*
n.d., not determined.

$^{NY}Fc\gamma RIIb^{-/-}_{B6}$ mice. In contrast, secondary IgG Ab responses were increased in both *Mb1Cre/*$^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ and *Cg1Cre/*$^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ mice compared with $^{NY}Fc\gamma RIIb^{fl/fl}_{B6}$ mice. This suggests that FcγRIIb is a B cell-intrinsic negative regulator of both primary and secondary IgG responses (110).

Although individually not sufficient to induce substantial autoimmunity, epistasis between the *Yaa* locus, the $^{Le}Fc\gamma RIIb^{-/-}_{B6}$ alleles and the C57BL/6 genome results in severe lupus-like disease (109) (**Figure 1**). The cell-type-specific role of FcγRIIb in this genetic disease model was studied (188). The *Yaa*$^{+}$*/CD19Cre/*$^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ mice developed milder lupus-like disease than *Yaa*$^{+}$/$^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mice similar to the disease in *Yaa*$^{+}$*/C/EBPα Cre/*$^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ mice whereas *Yaa*$^{+}$*/CD11cCre/*$^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ mice stayed disease free, like *Yaa*$^{+}$/$^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$ mice. This suggests that besides on B cells FcγRIIb on myeloid cells, but surprisingly not on DCs, contributes to the protection against spontaneous loss of immune tolerance in this mouse model. This confirms the observation with CIA in mice (110), discussed earlier, that FcγRIIb can be involved in different immune tolerance controlling mechanisms.

Strikingly, in the two strains with FcγRIIb deficient myeloid cells (*Yaa*$^{+}$/$^{Le}Fc\gamma RIIb^{-/-}_{B6}$ and *Yaa*$^{+}$*/C/EBPα Cre/*$^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$) but not in the strain with B cell-specific FcγRIIb deficiency (*Yaa*$^{+}$*/CD19Cre/*$^{Le}Fc\gamma RIIb^{fl/fl}_{B6}$) the frequency of peripheral Ly6C^{-}, but not Ly6C^{+} monocytes was increased. Monocytosis, an FcRγ dependent expansion of the monocyte compartment consisting mainly of Ly6C^{-} monocytes, is associated with the development of lupus nephritis in *Yaa*$^{+}$ lupus-prone mice. It has been reported that Ly6C^{+} monocytes mature in the circulation and are the precursors for Ly6C^{-} monocytes (189). Deficiency of FcγRIIb most likely accelerates the maturation of monocytes in *Yaa*$^{+}$/$^{Le}Fc\gamma RIIb^{-/-}_{B6}$ mice. Compared to Ly6C^{+} monocytes, mature Ly6C^{-} monocytes express significantly higher B cell-stimulating cytokines such as BSF-3, IL-10, and IL-1β, DC markers including CD11c, CD83, Adamdec1, and the anti-apoptotic factors Bcl2 and Bcl6. This makes monocytes the most promising FcγRIIb expressing candidate myeloid cells to modulate B cell tolerance (188, 190). The transcriptome of Ly6C^{-} monocytes suggests that they are long-lived and committed to developing into DCs.

Whether this monocyte-dependent tolerance breaking mechanism is unique for *Yaa*$^{+}$/$Fc\gamma RIIb^{-/-}_{B6}$ mice is not known but it is striking that also in SLE patients the serum levels of anti-dsDNA Abs highly correlate with the percentage of non-classical monocytes (191). Like mouse Ly6C^{-} monocytes, the human counterpart CD14^{low}CD16^{+} monocytes secrete high amounts of IL-1β in a TLR7-TLR8-MyD88–dependent manner (192).

CONCLUDING REMARKS

Forward and reverse genetics have provided convincing evidence that FcγRIIb is an important autoimmune susceptibility gene, involved in the maintenance of peripheral tolerance both in human and mice. In humans, a number of GWAS studies showed an association between a SNP *(rs1050501)* in the *FCGR2B* gene, causing a missense mutation (*FCGR2B*^{T232}) resulting in impaired FCGR2B function, and susceptibility to SLE. Meta-analyses confirmed that *FCGR2B*^{T232} homozygosity is one of the

strongest associations in SLE. Association of *rs1050501*with RA has also been reported.

In mice, the situation is more diffuse. Analysis of a variety of C57BL/6 mice congenic for the NZW and NZB haplotypes of *Fcγ RIIb*, with decreased expression, did not reveal clear unambiguous results with respect to the contribution of these haplotypes to the autoimmune phenotypes of these mice. The mechanism by which natural FcγRIIb variants contribute to autoimmunity is not well-understood.

The first $Fc\gamma RIIb^{-/-}$ mouse, generated by gene targeting in 129 derived ES cells and backcrossed into C57BL/6 background ($Fc\gamma RIIb^{-/-}_{129}$ mice), exhibited a surprisingly strong spontaneous autoimmune phenotype suggesting that FcγRIIb deficiency initiates loss of immune tolerance. However, independent studies with $Fc\gamma RIIb^{-/-}_{129}$ autoimmune V_H chain knockin mice pointed to a central role of FcγRIIb in a late immune tolerance checkpoint, that prevents autoimmunity by suppressing the production of autoreactive IgG from B cells, that escape negative selection in the GC and enter the AFC pathway. This should mean that FcγRIIb deficiency is mainly an amplifier of autoimmunity caused by other autoimmune susceptibility loci, rather than a primary initiator of the loss of immune tolerance. That was confirmed by the observation that $Fc\gamma RIIb^{-/-}$ mice on a pure C57BL/6 background ($Fc\gamma RIIb^{-/-}_{B6}$) have a much milder autoimmune phenotype than $Fc\gamma RIIb^{-/-}_{129}$ mice but when backcrossed into a mouse strain carrying the autoimmune susceptibility *Yaa* locus succumb to lupus-like disease. The strong autoimmune phenotype of the $Fc\gamma RIIb^{-/-}_{129}$ mouse could be explained by epistatic interactions between the C57BL/6 genome, the FcγRIIb KO allele and the 129 derived sequences (*Sle16*) flanking the *Fcγ RIIb* KO allele, containing the autoimmunity associated $Slamf_{129}$ (haplotype 2) gene cluster.

Spt-GC B and T_{FH} cells are activated, modestly (mainly B cells) in $Fc\gamma RIIb^{-/-}_{B6}$ mice, moderately in C57BL/6 $Slamf_{129}$ congenic mice and strongly in $Fc\gamma RIIb^{-/-}_{129}$ mice compared to Spt-GC B and T_{FH} cells in WT C57BL/6 mice. This was associated with a corresponding increase in ANA production, suggesting that FcγRIIb deficiency, besides enhancing autoimmunity caused by other autoimmune susceptibility loci, might play a modest role in the induction of the loss of immune tolerance in the GC, explaining the development with low penetrance of low ANA titers in $Fc\gamma RIIb^{-/-}_{B6}$ mice. An alternative explanation is that the low ANA titers in $Fc\gamma RIIb^{-/-}_{B6}$ mice reflect the natural background of autoreactive B cells in the GC that are prevented to enter the AFC pathway in the presence of FcγRIIb. The analysis of the development of self-reactive GC B cells and plasma cells by large scale Ig cloning from single isolated B cells, as performed with $Fc\gamma RIIb^{-/-}_{129}$ mice, should be repeated in $Fc\gamma RIIb^{-/-}_{B6}$ mice, to determine how FcγRIIb deficiency influences the frequency at which autoreactive ANA-expressing B cells participate in GC reactions, and develop in plasma cells, under physiological conditions, without the confounding effect of $Slamf_{129}$ expression.

Studies with cell-type-specific FcγRIIb deficient mice revealed that besides on B cells, FcγRIIb on DCs and monocytes can also contribute to the maintenance of immune tolerance, indicating that FcγRIIb is involved in different immune tolerance maintaining mechanisms. Series of observations suggest that on B cells impaired FcγRIIb function effects not only antibody titers but also affinity maturation and memory responses of B cells and plasma cell homeostasis associated with an increase in the production of autoantibodies. However, the underlying cellular and molecular mechanisms are not well-understood. Most likely new model systems including adoptive cell transfer and tools such as cell type-specific KO mice, to study the GC reaction, are required to answer these questions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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There Is (Scientific) Strength in Numbers: A Comprehensive Quantitation of Fc Gamma Receptor Numbers on Human and Murine Peripheral Blood Leukocytes

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Antibodies are essential mediators of immunological defense mechanisms, are clinically used as therapeutic agents, but are also functionally involved in various immune-mediated disorders. Whereas IgG antibodies accomplish some of their biological tasks autonomously, many functions depend on their binding to activating and inhibitory Fcγ receptors (FcγR). From a qualitative point of view expression patterns of FcγR on immunologically relevant cell types are well-characterized both for mice and humans. Surprisingly, however, there is only quite limited information available on actual quantities of FcγR expressed by the different leukocyte populations. In this study we provide a comprehensive data set assessing quantitatively how many individual human and mouse FcγRs are expressed on B cells, NK cells, eosinophils, neutrophils, basophils and both classical, and non-classical monocytes under steady state conditions. Moreover, among human donors we found two groups with different expression levels of the inhibitory FcγRIIb on monocytes which appears to correlate with haplotypes of the activating FcγRIIIa.

Keywords: Fc receptors, antibodies, human leukocytes, murine leukocytes, quantification, receptor numbers, neutrophils, monocytes

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INTRODUCTION

Antibodies of the immunoglobulin G (IgG) isotype are essential mediators of immunological defense mechanisms. Whereas they carry out some of their biological functions autonomously, such as blocking of cell surface receptors, most of their functions depend on the binding of the antibody Fc-domain to specific receptors, the Fc gamma receptors (FcγR). Whereas binding of immune complexes (IC) is possible to all FcγR including the low/medium receptors FcγRIIa and FcγRIIb, FcγRIIIa and FcγRIIIb in man and FcγRIIb, FcγRIII and FcγRIV in mice, monomeric IgG molecules can only bind to human or murine high affinity receptor FcγRI [reviewed e.g., in (1–3)].

The repertoire of murine and human FcγR includes the inhibitory FcγRIIb, which contains an intracellular immunomodulatory tyrosine based inhibitory motif (ITIM) whereas most other FcγR carry an activating ITAM motif. These motifs are either located in the same polypeptide chain as the ligand binding domain (FcγRIIb and FcγRIIIb) or on an accessory Fc receptor gamma chain. One exception is the human FcγRIIIb which contains no transmembrane or intracellular polypeptide domain, but is rather linked to the cell membrane by a GPI anchor (4).

Immune cell populations are characterized by a typical expression pattern of FcγR. Whereas most of them express both activating and inhibitory FcγR, B cells only express the inhibitory FcγRIIb, which regulates the activity of the B cell receptor. Natural Killer (NK) cells on the other side selectively express the activating FcγRIII. Thus, from a qualitative point of view expression patterns of FcγR on different immune cell subsets are well-characterized both, for mice and man (1–3, 5–12).

For many cell types, their functional response to external signals is accompanied by pronounced up- or down-regulation of various surface receptors. This clearly shows that the amount of any given surface receptor on a cell is an important characteristic for its physiological functionality. The outcome of immune complex binding to a cell is determined by the sum of activating and inhibitory signals triggered through the respective activating and inhibitory FcγRs. One factor influencing these signals is the affinity of the respective IgG isotype to the distinct FcγRs present on the cell surface. This led to the development of the concept of the so called A/I ratio as a prediction of the outcome of binding of IgG molecules of a given isotype based on its affinities to the inhibitory and activating receptors, respectively (13–15). Based on the A/I ratio concept mathematical models have been developed predicting IgG activity (16). However, it is to be expected that other factors such as the avidity of the immune complex which is influenced by the number of immunoglobulins bound to the antigen and the number of activating and inhibitory receptors on the surface of the respective effector cell may influence IgG effector functions (17). Thus, knowledge of the quantities of the various FcγR on the cell surface of immune cell subsets may help to develop more precise models to understand how IgG antibodies trigger cellular responses.

Whereas FcγR expression is rather well-described qualitatively, there is only quite limited information available on actual numbers of FcγR expressed by the different leukocyte populations (18–35). To our knowledge there is no publication, where such numbers have been determined for the main leukocyte populations in parallel, even though Antal-Szalmas et al. (18) and the group of Guyre (19, 22–25) provided data on human neutrophils and monocytes. In addition, work on FcγR cell surface numbers often dates back to times, where certain cell subpopulations with an entirely different FcγR repertoire (such as classical and non-classical monocytes), had not been distinguished.

Flow cytometry is a common method for the analysis of cell surface receptors. Qualitative comparison of the relative expression of a given cell surface receptor on different cell populations based on the fluorescence intensities mediated by binding of fluorochrome-labeled antibodies specific for this receptor is rather straightforward. In contrast, quantitative comparison of different receptors on the same or different cell populations by simple comparison of fluorescence intensities is not possible since there are inherent variations between the different anti-receptor antibody conjugates with respect to their specific fluorescence (i.e., fluorescence intensity per molecule). This is obvious when conjugates with different fluorochromes are used. However, even antibodies conjugated with the same fluorochrome may differ in the stoichiometry of fluorophores per antibody molecule. This variability can be partially reduced by using conjugates with very large fluorophores like R-Phycoerythrin (PE), where usually not more than one fluorochrome can be linked to each antibody molecule (36). In spite of this advantage of PE studies by David et al. suggest that there is also a variation in the quantum yield of PE from various organisms (37). The information, from which species the used PE is derived from, is usually not available from the antibody companies or providers of labeling kits. In addition, also for PE the stoichiometry of fluorophores per antibody molecule for any lot of antibody conjugate may be <1 due to partial degradation or incomplete coupling.

To achieve a more definitive estimate of receptor numbers per cell the establishment of reference curves comparing the observed fluorescence to the fluorescence of well-defined reference beads appears to be better suited. Thus, we used sets of commercially available beads with distinct numbers of binding sites for anti-FcγR antibodies, respectively, and established reference curves for all anti-FcR monoclonal antibody (mAb) conjugates used in this study. Based on these references we present here the ABC values (i.e., the *a*ntibody-*b*inding *c*apacity for respective anti-FcγR antibodies) for all activating and inhibitory FcγR on the main leukocyte populations in peripheral blood of C57BL/6 and FcγR knockout mice and in healthy human volunteers under steady state conditions. This includes Natural Killer (NK) cells, B cells, T cells, neutrophils, eosinophils and basophils as well as classical and non-classical monocytes. These monocyte subsets have comparable population size in murine blood. In contrast, in humans the classical monocytes represent the vast majority of monocytes whereas non-classical monocytes represent only a small fraction of the monocyte population but nonetheless exert important biological functions. In both species these subsets have differential FcγR repertoires and, thus, specific effector functions upon engagement with immune complexes [see e.g., (38)].

MATERIALS AND METHODS

Mice

Female mice at 8–16 weeks of age on C57BL/6 background were used in all experiments. C57BL/6J mice (JAX strain 000664) were purchased from Janvier (Le Genest-Saint-Isle, France). FcγRI deficient mice (39) were originally provided by M. Hogarth (JAX number: not available (N/A)), FcγRIIb deficient [(40), JAX number: N/A], FcγRIII deficient (JAX number N/A) and FcγRIV deficient [(41), JAX number: N/A] mice by J. Ravetch. Mice were kept in the animal facilities of Friedrich-Alexander-University Erlangen-Nürnberg under specific pathogen-free conditions in individually ventilated cages according to the guidelines of the National Institutes of Health and the legal requirements in Germany. Animal experiments conducted in the animal facility of the FAU were approved by government of lower Franconia.

Human Donors

For the characterization of human leukocytes venous blood of male and female healthy adults was used. The use of human material for scientific purposes was carried out in accordance

with the recommendations of and approved by the ethics committee of the Friedrich-Alexander University Erlangen-Nürnberg. All subjects gave written informed consent regarding usage of their biological material for the scientific research presented here.

Preparation of Murine and Human Peripheral Blood Leukocytes

For isolation of murine PBLs blood was drawn from the retro-orbital plexus using anti-coagulant micro hematocrit capillaries. Human PBLs were isolated from venous blood of male and female healthy human adults using anticoagulant EDTA Monovettes. Erythrocytes from both murine and human blood were lysed using deionized H_2O and subsequent restoration of iso-osmolality. After repeated washing in cold FACS buffer containing sodium azide to inhibit changes in the surface presentation of proteins, cells were continued processing for flow-cytometric analysis.

Flow Cytometry

Characterization of Murine PBLs

Single cell suspensions with typically 1–2 × 10^5 cells per sample were usually incubated for 15 min on ice with Fc-block antibodies to minimize unspecific binding to Fc receptors, followed by staining with fluorochrome-coupled antibodies for ∼20 min. Since single FcγRs were to be stained specifically, full Fc block was inconvenient. We, thus, pretreated cells with anti-CD16/32 clone 2.4G2 to block FcγRII and III only when FcγRIV was quantified. This Fc block was not used in analyses of FcγRI, since 2.4G2 may also block high affinity receptor FcγRI via its Fc-part on cells were the antibody is bound in cis to FcγRII or III (42). Since we have recently shown that also medium-affinity receptor FcγRIV can bind the Fc-part of several rat and mouse IgG subclasses and cause false positive results in flow cytometry (43), FcγRIV was blocked by clone 9E9 in all analyses where other receptors than FcγRIV were to be analyzed.

For the identification of cell populations we used antibodies against the following antigens: B220 (clone RA3-6B2, APC conjugated, BD Biosciences or FITC conjugated Biolegend), CD3e (clone 145-2C11, FITC conjugated, Biosciences and Biolegend or BV510 or AlexaFluor 647 conjugated, Biolegend), CD11b (clone M1/70, PerCP-Cy5.5 conjugated), CD19 (clone 6D5, BV510 conjugated, Biolegend), CD45 (clone 30-F11, APC-Cy7 conjugated BD Biosciences and Biolegend or APC-Fire750 conjugated, Biolegend), CD49b (clone DX5, APC conjugated, BD Biosciences), CD62L (clone MEL-14, PE-Cy7 conjugated, Biolegend), Gr1 (clone RB6-8C5, APC- or AlexaFluor 647 conjugated, BD Biosciences and Biolegend or BV510 conjugated, Biolegend), IgE (clone R35-72, FITC conjugated, BD Biosciences), Ly6G (clone 1A8, FITC-conjugated, provided by BD Biosciences and Biolegend), NK1.1 (clone PK136, FITC conjugated, Southern Biotech, BD Biosciences and Biolegend), and TCRß (clone H57-597, FITC conjugated, Biolegend). Flow cytometric measurements were carried out on a FACSCanto II (BD Bioscience). Briefly, cell aggregates were excluded by their light scatter characteristics. Dead cells were excluded from analysis using 4′,6- diamidino-2-phenylindole (DAPI). Single viable $CD45^+$ leukocytes were divided into SSC^{high} granulocytes and further distinguished into neutrophils and eosinophils by Ly6G. Among SSC^{low} cells NK cells were characterized as $NK1.1^+$ $CD11b^{intermediate}$ and monocytes as $NK1.1^-$ $CD11b^{high}$. Monocytes were further distinguished by expression of CD62L and Gr1 (high for classical monocytes and negative-low for non-classical monocytes). Among SSC^{low} CD11b negative cell B cells were characterized by binding of anti-B220 whereas T cells were B220 negative and, in addition, in some experiments were characterized as positive for TCRß or CD3e. Murine basophils were identified as cells with low side-scatter characteristics which were negative for lineage markers CD19, CD3 and Gr-1 but were $CD49b^+$ and positively stained for IgE. A representative gating for murine leukocytes is shown in **Supplementary Figure 1**.

Characterization of Human PBLs

Antibodies detecting the following antigens were used for the characterization of human leukocyte populations: CD3 (clone SK7, PE-Cy conjugated, BD Biosciences), CD14 (clone HCD14, FITC conjugated and clone M5E2, PerCP-Cy5.5 conjugated, Biolegend), CD16 (clone 3G8, PE-Cy7 conjugated, BD Biosciences), CD19 (clone HIB19, APC conjugated, BD Biosciences and Biolegend), CD33 (clone WM53, BV510 conjugated, Biolegend), CD45 (clone HI30, APC-Fire750 or APC-H7 conjugated, Biolegend), CD56 (clone MEM188, PerCP-Cy5.5 conjugated and FITC conjugated, Biolegend), CD123 (clone 6H6, PE-Cy7 conjugated, Biolegend), FcεRIA (clone AER-37 (CRA-1), PerCP-Cy5.5 conjugated, Biolegend), HLA-DR (clone L243, APC conjugated, Biolegend). Per sample typically 1–2 × 10^5 human leukocytes were used.

To avoid unspecific binding of antibodies for the quantification of human Fc receptors to any other FcγR via their Fc part, we utilized the Human TruStain FcX™ Fc Receptor Blocking Solution (Biolegend). This Fc-block protects from Fc-mediated binding to FcγRs by pre-occupying their Fc binding sites, but does not inhibit antigen-specific detection of anti-huFcγR antibodies which takes place with higher affinity. In pretests we verified that this reagent did not affect antigen-specific FcγR-detection, prior to its employment in quantification experiments (**Supplementary Figure 2**). FcγRIIb quantification took additional advantage from the fact that the anti-FcγRIIb antibody is a 2B6-variant where N-glycosylation is prevented. Since binding to FcγRs via the Fc-part is impaired for a-glycosylated antibodies (44, 45), undesirable Fc-mediated binding of 2B6 to other Fc receptors is prevented. Flow cytometric measurements were carried out on a FACSCanto II (BD Bioscience). Briefly, cell aggregates were excluded by their light scatter characteristics and dead cells were excluded from analysis by DAPI staining.

Among $CD45^+$ leukocytes, neutrophils and eosinophils were identified by their high granularity resulting in their distinct light scatter characteristics (SSC^{high}) and distinguished by the CD16 expression of neutrophils and/or by the intrinsic auto-fluorescence of the eosinophils. Among SCC^{low} cells NK cells were gated as being CD56 positive but negative for CD14 and CD33. Monocytes were identified in the $CD56^-$ population by the expression of CD33 and CD14. They were further

distinguished as classical $CD14^{high}$ $CD16^{low}$ and much less frequent non-classical $CD14^{low}$ $CD16^{+}$ monocytes. Within the CD33- CD14- population B cells were identified by their expression of CD19 and T cells by the expression of CD3 in absence of CD56. Basophils were identified as SSC^{low} $CD45^{dim}$ $CD123^{+}$ HLA-DR negative cells, which were positively stained for IgE receptor FcεR1. In addition to leukocytes we analyzed human platelets, which were characterized by their small size as reflected by low light scatter and by their expression of CD41a. A representative gating strategy for human leukocytes and platelets is shown in **Supplementary Figure 3**.

Quantification of Fc Receptors

Fcγ receptors on leukocytes were quantified by measuring their Antibody Binding Capacity (ABC) for antibodies specific for the respective FcγR. ABC values on leukocytes were calculated using a specific reference curve for the correlation between fluorescence intensity of a cell upon binding by the respective fluorochrome-conjugated anti-FcγR antibody and the number of antibody binding sites. These reference curves were generated using sets of Quantum Simply Cellular (QSC) microspheres (Bangs Laboratories Ltd.) with known numbers of antibody binding sites as provided by the manufacturer. Beads and cells were stained with the same concentration of the respective anti-FcγR antibodies. Reference curves were established in each experiment for the analyzed anti-FcγR antibodies. According to manufacturer's instructions a titration curve should be prepared for every quantitating antibody using the QSC beads to determine its saturating concentration. However, earlier experiments suggested that this concentration might not be sufficient to saturate all binding sites on cells for all antibodies. Insufficient saturation of binding sites on cells with full saturation on reference beads will lead to an underestimation of the number of binding sites on the target cells. The difference between saturating concentrations for cells and QSC beads is easily conceivable since binding to cells is achieved via antigen-specific binding domains in the Fab regions whereas binding to beads is achieved via other antibody domains probably via the Fc-region and binding to both entities may take place with very different binding affinities. We, thus, suggest titrating quantitating antibodies both for binding to cells and to QSC beads in order to aim at saturating binding to both entities.

The following PE-conjugated anti-FcγR antibodies were used for mouse receptor quantification: anti-msFcγRI/CD64 mouse IgG1 clone X54-5/7.1 (BD Biosciences), anti-msFcγRIIb/CD32b mouse IgG2a clone Ly17.2 (in-house production and labeling), anti-msFcγRIII/CD16 rat IgG2a clone 275003 (R&D Systems), anti-msFcγRIV Arm. hamster IgG clone 9E9 (in-house production and labeling or Biolegend).

PE-labeled antibodies specific for human FcγR were: anti-huFcγRI/CD64 mouse IgG1 clone 10.1 (BD Biosciences), anti-huFcγRIIb/CD32B humanized IgG1 clone 2B6 (in-house production and labeling) and anti-huFcγRIII/CD16 mouse IgG1 clone 3G8 (Biolegend). Since no fully FcγRIIa/CD32A-specific mAb was at hand, expression of this receptor was analyzed by staining with a anti-CD32 antibody (clone IV.3, antibodies-online GmbH) after pre-blocking of CD32B with clone 2B6 N297Q which recognizes specifically FcγRIIb but not FcγRIIa (46). Among anti-human FcγRII antibodies IV.3 appears to be one of the clones with the most preferential binding to FcγRIIa over FcγRIIb. However, under conditions of saturating binding to FcγRIIa expressing leukocytes, which are important for reliable quantification of antibody-binding sites by this method, even IV.3 reveals pronounced binding to FcγRIIb e.g., on B cells (**Supplementary Figure 4**). Binding of IV.3 to FcγRIIb can be efficiently blocked by pre-treatment with anti-FcγRIIb antibody 2B6 (10 μg/ml) without affecting binding to highly FcγRIIa-expressing monocytes (**Supplementary Figure 4**). Thus, pre-blocking of FcγRIIb is a versatile and necessary step for reliable quantification of FcγRIIa expression by IV.3.

Human FcγRs I and III could be detected directly by antibody clones 10.1 and 3G8, followed by using anti-mouse IgG QSC beads. In a similar manner, human FcγRIIb was detected with the antibody clone 2B6 and by using anti-human IgG QSC beads. To quantify FcγRIIb we used a PE-conjugated recombinant 2B6 whose Fc part is of human origin. Thus, we employed Quantum™ Simply Cellular® (QSC) anti-human beads to establish a reference curve for 2B6 binding sites.

Anti-FcγR antibodies were purchased from BD Biosciences, BioLegend, R&D Systems Europe or prepared in house. To minimize potential systematic variations in the quantification of different receptors by engagement of different fluorophores we used a single type of fluorophore. We chose Phycoerythrin (PE) for this purpose, since due to its size there is typically one fluorochrome conjugated to each antibody molecule, thereby minimizing variations in specific fluorescence and it lacks the pronounced self-quenching capacity of fluorochromes like FITC (47). Anti-FcγR antibodies were either purchased pre-labeled or were conjugated in-house. According to the host species of the respective anti-FcγR antibody, we used anti-mouse IgG, anti-rat IgG or anti-human IgG QSC beads following manufacturer's instructions. Since anti-FcγRIV is derived from Armenian hamster and no QSC beads specifically binding antibodies of this species are available, we performed a sandwich-assay where anti-mouse IgG beads were pre-coated with mouse anti-hamster antibody. The formulation of the latter is a commercially available equal mixture (BD Biosciences) of two murine antibody clones specific for either hamster IgG1 or hamster IgG2-3, respectively. The anti-FcγRIV clone 9E9 is an Armenian hamster IgG not further characterized regarding the IgG subtype. It should, thus bind to one of these two mouse IgG clones. Assuming that upon loading QSC microspheres with this mixture half of the anti-mouse IgG binding sites on the microspheres are loaded with the antibody clone which binds 9E9 and each of these antibody molecules has two binding sites for 9E9 we assumed the capacity of the QSC microspheres for 9E9 binding to be equal to the mouse IgG binding capacity of these microspheres as provided by the manufacturer.

To enable subtraction of ABC-background values based on background fluorescence of the respective cells, we used FMO ("fluorescence-minus-one") controls in each experiment, where cells were stained with all antibodies except the anti-FcγR antibody. Flow cytometric analysis was done on a FACS Canto II (BD Biosciences, Heidelberg). Data were analyzed

with FACSDiva Software (BD). For ABC calculation we used QuickCal® software provided by Bangs Laboratories.

An example for the quantification procedure from flow cytometric analysis to calculation of ABC values is provided in **Supplementary Figure 5**.

Identification of Allelic Variants for Human Donors

For the identification of FcγR haplotypes of human donors, genomic DNA was isolated from peripheral blood and stored at −20°C. For genotyping of FcγRIIb$^{232I/T}$ und FcγRIIb$^{G/C-386/A/T-120}$ alleic variants a nested PCR was carried out with an initial long-range PCR (93°C 15″, 68°C 17′ for 10 cycles; 93°C 15″, 68°C 28′ for 28 cycles; PCR product ∼17 kbp) using primers LR-FOR (5′ ctccacaggttactcgtttctaccttatcttac 3′ and LR-REVERSE (5′ gcttgcgtggcccctggttctca 3′) PCR products were extracted from Agarose gels and used as template for a second PCR, either using primer pair PP-FOR (5′ caatttaccgagagcaagacagc 3′) and PP-REVERSE (5′ gcagtcagcccagtcactctcagt 3′) for amplification of promoter polymorphism alleles (95°C 30″, 58° 30″, 72°C 90″ for 35 cycles; final elongation 72°C 5′; PCR product 1946 bp) or primer pair I232T-FOR (5′ cctgcctgctcacaaatgta 3′) and I232T-REVERSE (5′cactgctctccccaagac 3′) for amplification of alleles for the I232T transmembrane polymorphisms (98°C 15″, 58°C 20″, 72°C 30″ for 35 cycles; final elongation 72°C 7′; PCR product ∼750 bp). Products of both PCRs were eluted from agarose gels and sequenced by GATC Biotech using primer PPseq (5′ tgacatacctccttgtccttgtt 3′) for the promoter polymorphism and I232T-FOR for the polymorphism in the transmembrane region.

Polymorphisms of FcγRIIa$^{131H/R}$ and FcγRIIIa$^{158V/F}$ were characterized by nested allele-specific PCR. For FcγRIIa$^{131H/R}$ an initial PCR (94°C 5′, 56°C 5′, 72°C 5′ for 10 cycles, followed by 94°C 60″, 56°C 60″, 72°C 2′ for 30 cycles, final elongation 72°C, 10′) with primer pairs IIa-1st FORWARD (5′ ggagaaaccatcatgctgag 3′) and IIa-1st REVERSE (5′ gaagagctgcccatgctg 3′) was performed to amplify a *fcgr2a* specific DNA fragment containing the polymorphism. This was followed by an allele-specific PCR with common IIa131-REVERSE primer (5′ caattttgctgctatgggc 3′) and either FORWARD primer IIa131H (5′ gaaaatcccagaaatttttcca 3′) or IIa131R (5′ gaaaatcccagaaatttttccg 3′) which could only amplify the histidine or arginine variant, respectively. Successful PCR reactions provided a 249 bp fragment. For FcγRIIIa$^{158V/F}$ an initial PCR (94°C 5′; followed by 94°C 60″, 58°C 30″, 72°C 2.5′ for 30 cycles, final elongation 72°C, 10′) with primer pairs IIIa-1st FORWARD (5′ gtgtctttcaggctggctg 3′) and IIIa-1st REVERSE (5′ gaccagaatagtttaatctcg 3′) was performed to amplify a *fcgr3a* specific DNA fragment containing the polymorphism. This was followed by an allele-specific PCR with common IIIa158-FORWARD primer (5′ tcacatatttacagaatggcaaagg 3′) and either REVERSE primer IIIa158V (5′ tctctgaagacacatttctactccctac 3′) or IIIa158F (5′ tctctgaagacacatttctactccctaa 3′) which could only amply the histidine or arginine variant, respectively. Successful PCR reactions provided a 138 bp fragment.

Statistics

Panels with data sets for analysis of statistical significance are depicted as bar charts. Data in bar charts are expressed as mean + standard deviation if all data sets are normally distributed and as median ± interquartile range (IR) if at least one data set in the panel is not normally distributed according to Shapiro-Wilk test. Otherwise, sets data are provided either in box plots or as individual data points. Statistical significance of differences between medians of two sets of data was analyzed by Mann-Whitney test. Data were analyzed and plotted with Graph Pad Prism software (GraphPad Software Inc., San Diego, CA).

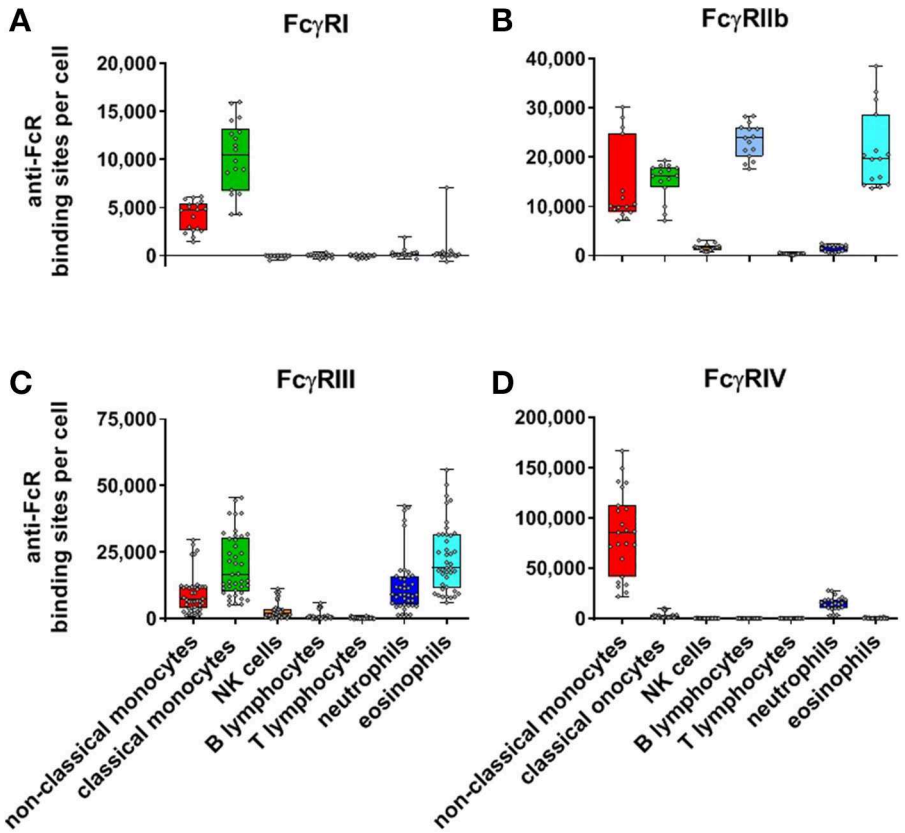
RESULTS

In the present work we analyzed the expression of Fcγ receptors I, IIb, III, and IV on murine peripheral blood leukocytes and Fcγ receptors I, IIa, IIb, and IIIa/b on human peripheral blood leukocytes by flow cytometric analysis with fluorochrome-conjugated antibodies against the various Fcγ receptors (anti-FcR). FcγR expression on cells is referred to as their antibody binding capacity (ABC, depicted as *antibody-binding sites per cell*) for the respective anti-FcR antibodies. To achieve this, fluorescence intensity of anti-FcR antibody bound cells and cells from FMO controls without anti-FcR antibody was translated into ABC, using reference curves that were established in each experiment for all tested anti-FcR antibodies using Quantum™ Simply Cellular® (QSC) microspheres. Anti-mouse, anti-rat or anti-human QSC microspheres (Bangs Laboratories Ltd.) were used according to the host species of the respective anti-FcγR antibody.

Quantification of Mouse Fcγ Receptors

Using this methodology, we quantified the ABC for murine Fcγ receptors I (CD64), IIb (CD32b), III (CD16), and IV using PE-labeled anti-FcγR antibodies on peripheral blood leukocytes of C57BL/6 mice under steady state conditions. Quantification of mouse FcγRs was done repeatedly over several years by different researchers and by using different lots of QSC beads, antibodies and antibody providers. The summary of these measurements are depicted in **Figure 1**. In contrast to merely qualitative analyses our quantitative approach also allows direct comparison of ABC values for activating vs. inhibitory receptors on the different cell types that co-express both receptors.

As shown in **Figure 1**, expression of the high affinity FcγRI was restricted to monocytes, with classical monocytes expression roughly twice as much FcγRI than non-classical monocytes. With 24,000 ABC per cell, the inhibitory FcγRIIb was expressed most strongly on B cells, closely followed by eosinophils with 20,000 ABCs. On eosinophils FcγRIIb expression levels were matched by a nearly similar expression of activating FcγRIII (1.9 × 10^4 binding sites). Co-expression of inhibitory and activating receptors was also found on mouse monocytes. On classical monocytes FcγRIIb (1.6 × 10^4 binding sites) expression levels faced a slightly higher number of activating receptors comprising mainly of FcγRIII (1.7 × 10^4 binding sites) and FcγRI (1 × 10^4 binding sites), but also some FcγRIV (2 × 10^3 binding sites). This FcγRIV expression may be due to some classical monocytes



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median ABC [x10^3 binding sites/cell]	FcγRI	FcγRIIb	FcγRIII	FcγRIV
non-classical monocytes	5	10	7	86
classical monocytes	10	16	17	2
NK cells	<1	2	2	<1
B lymphocytes	<1	24	<1	<1
T lymphocytes	<1	<1	<1	<1
neutrophils	71	2	9	15
eosinophils	<1	20	19	<1

FIGURE 1 | Fc gamma receptors on murine peripheral blood leukocytes. Depicted are box plots showing anti-FcR binding sites for **(A)** FcγRI, **(B)** FcγRIIb, **(C)** FcγRIII, and **(D)** FcγRIV on indicated leukocyte subsets together with **(E)** a tabular presentation of the median number of binding sites. n = 15–41 from 4 to 11 independent experiments.

upregulating FcγRIV in the process differentiation into non-classical monocytes (48, 49). For FcγRIV on non-classical monocytes, we calculated the highest expression of all murine FcγRs (8.6 × 10^4 binding sites). In contrast to this high FcγRIV expression, moderate levels of activating FcγRs FcγRI (5 × 10^3 binding sites) and FcγRIII (7 × 10^3 binding sites) were noted. The inhibitory FcγRIIb was only expressed at intermediate levels on this monocyte subset (1 × 10^4 binding sites) and remained about one order of magnitude below activating FcγR numbers.

Low values of anti-FcγRIIb binding sites per cell have been measured on NK cells and neutrophils (both 2 × 10^3 binding sites), as well as FcγR-lacking T-cells (<1 × 10^3 binding sites)

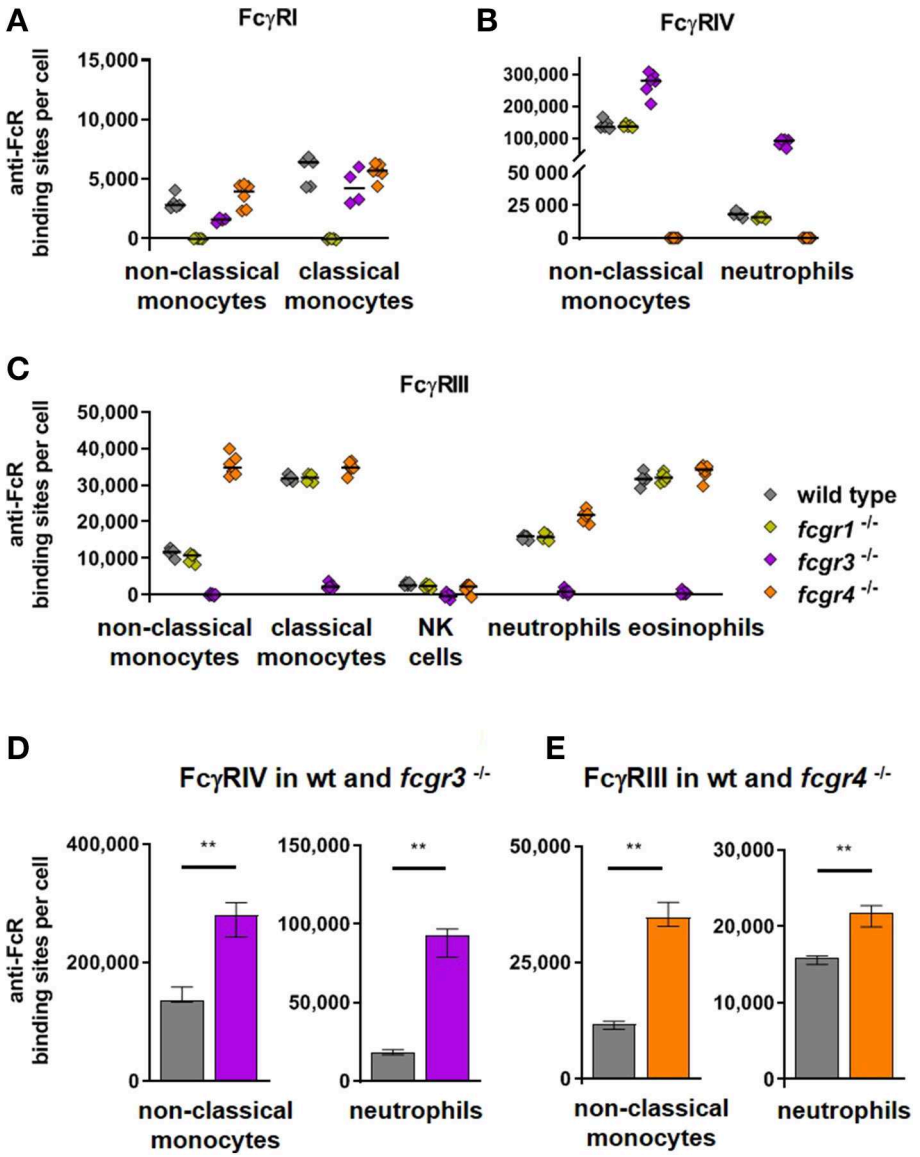


FIGURE 2 | Activating Fc gamma receptors on PBLs of wild type and activating FcγR knockout mice. In **(A–C)** individual values of anti-FcγR binding sites on cell populations prominently expressing the respective receptors are depicted for C57BL/6 wt mice and knockout mice lacking FcγRI, FcγRIII, or FcγRIV. Data are shown **(A)** for FcγRI on monocytes, **(B)** for FcγRIV on non-classical monocytes and neutrophils and **(C)** for FcγRIII on monocytes, NK cells, neutrophils and eosinophils. In **(D–E)** the anti-FcγR binding sites per cell are show for groups with prominently increased activating receptors on knockout compared to wild type mice, i.e., in **(D)** for FcγRIV in *fcgr3*$^{-/-}$ knockout mice (violet bars) and in **(E)** for FcγRIII in *fcgr4* $^{-/-}$ knockout mice (orange bars) in comparison to C57Bl/6 wild type mice (gray bars) on non-classical monocytes and neutrophils. $n = 5$–6; median ± IR; Mann-Whitney test for significance; $^{**}p < 0.01$.

upon staining with self-labeled anti-FcγRIIb-PE. However, in comparison to cell subsets known to express the inhibitory FcγRIIb, such as monocytes, eosinophils, and B-cells these values—especially for T cells—appear negligible. At this point, however, we cannot explicitly distinguish whether there is in fact very low FcγRIIb expression at least on NK cells and neutrophils, or whether for example some free PE molecules from the Ly-17.2 in-house labeling were capable of binding to the cells. Even if the low expression of the inhibitory FcγRIIb on neutrophils was indeed real under steady state conditions, it faces pronounced expression of activating FcγRIV (1.5×10^4 binding sites) and moderate levels of FcγRIII (9×10^3 binding sites) on these cells. Finally, we could verify our previously published observation that murine NK cells express only low levels of FcγRIII (50) (2×10^3 binding sites) during the steady state.

FcγR Expression by FcγR Knockout Mice

It has been reported previously that deficiencies in FcγR expression may modify the expression of other FcγRs. For example FcγRIV expression on neutrophils was increased in

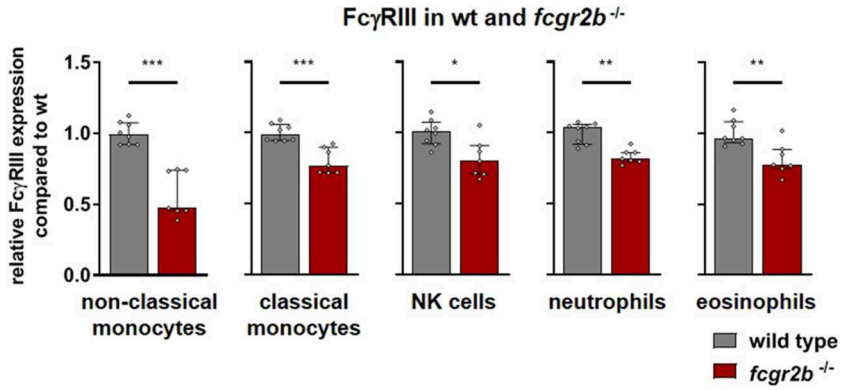


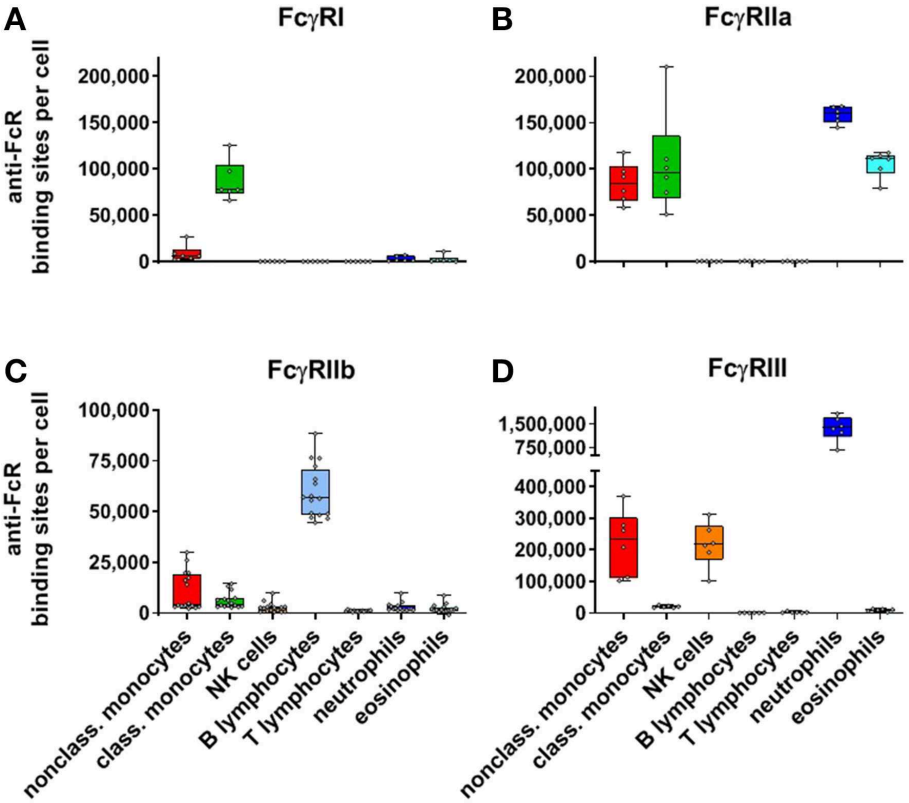
FIGURE 3 | Activating Fc gamma receptors on PBLs of wild type and inhibitory FcγR knockout mice. Depicted are the anti-FcγRIII binding sites per cell for wild type (gray bars) and *fcgr2b*$^{-/-}$ knockout mice (red bars) from two experiments, normalized to the mean expression in the respective wild type cohort. $n = 7$–8; Shown is the median ± IR together with the individual values; Mann-Whitney test for significance; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

FcγRIII knockout mice (51–53), but not in FcγRI deficient mice (51, 53). In addition we had observed that deletion of FcγRIV resulted in a slight up-regulation of FcγRIII on neutrophils (52). These results were corroborated in the present work where we analyzed potential compensatory effects upon deficiency of single activating receptors in a parallel analysis of C57BL/6, FcγRI-, FcγRIII- and FcγRIV- knockout mice. **Figure 2** depicts anti-FcR binding sites for FcγRI on monocytes (**Figure 2A**), and FcγRIV on non-classical monocytes and neutrophils (**Figure 2B**) (**Supplementary Figure 6** for other cell types also) as well as FcγRIII on monocytes, NK cells, neutrophils and eosinophils (**Figure 2C**) for C57BL/6 mice and knockout mice lacking FcγRI, FcγRIII, or FcγRIV. This analysis revealed that FcγRIV expression was significantly increased by nearly a factor of two on non-classical monocytes of FcγRIII deficient mice (2.8×10^5 antibody binding sites per cell) compared to C57BL/6 mice (1.4×10^5 binding sites) (**Figure 2D**). On neutrophils FcγRIII deficiency increased FcγRIV expression even about 5-fold (9.3×10^4 in FcγRIII deficient mice vs. 1.8×10^4 in wt mice). Among FcγRIII expressing cells lack of FcγRIV resulted in a significant increase of FcγRIII on neutrophils (2.2×10^4 in FcγRIV deficient mice vs. 1.6×10^4 in wt mice) and 3-fold higher ABC values on non-classical monocytes (3.5×10^4 in FcγRIV deficient mice vs. 1.2×10^4 in wt mice) (**Figure 2E**).

In FcγRIIb deficient mice the activating FcγRIII was moderately down regulated compared to wild type mice on most cell subsets (**Figure 3**). On non-classical monocytes, however, FcγRIII expression was reduced by about one half according to the combined results of two independent experiments (**Figure 3**). FcγRI and FcγRIV appeared unaffected by the FcγRIIb knockout (**Supplementary Figure 7**).

Quantification of Human Fcγ Receptors

In addition to quantifying mouse FcγR-expression, we also assessed human FcγR expression. Thus, we quantified expression levels for inhibitory FcγRIIb and the closely related but ITAM-bearing activating FcγRIIa on human leukocytes. We also studied but did not experimentally distinguish both variants of human FcγRIII, i.e., FcγRIIIa with a canonical polypeptide transmembrane domain and the GPI-anchored FcγRIIIb. As the latter receptor is exclusively expressed on neutrophils, and at low levels on basophils (54), however, this should not impact on the reported numbers. The calculated anti-FcR binding sites for the human Fc receptors are depicted in **Figure 4**. In the literature, FcγR expression has been mainly studied on human neutrophils and monocytes by various methods (18–34). According to our results, FcγRI is barely expressed by neutrophils in steady-state, consistent with observations by others (18–29). On monocytes, FcγRI expression was described with numbers ranging from about 1–4 $\times 10^4$ anti-FcγRI or monomeric IgG binding sites (18, 19, 22, 23, 27, 30–33), but in contrast to our work these studies did not distinguish between monocyte subsets. As shown in **Figure 4A** non-classical monocytes have low to moderate FcγRI expression (6×10^3 anti-FcγRI binding sites). In contrast, classical monocytes ($\sim 8 \times 10^4$ binding sites) expressed considerably higher numbers of FcγRI. The same was true for FcγRII where numbers between 2 and 4.7×10^4 molecules per cell have been published (18, 19, 22, 23), without differentiating between activating and inhibitory FcγRII receptors or between classical and non-classical monocytes. In our study, we calculated for classical monocytes $\sim 1 \times 10^5$ binding sites for FcγRIIa and 4×10^3 binding sites for FcγRIIb whereas non-classical monocytes had ABC values of $\sim 8 \times 10^4$ binding sites for FcγRIIa and 4×10^3 binding sites for FcγRIIb. For neutrophils, where we calculated 1.6×10^5 anti-FcγRIIa binding sites but barely any FcγRIIb expression (2×10^3 binding sites), published pan-FcγRII values are in the range of ~ 1–4.5×10^4 (18, 19, 22–25, 34). The ABC values for the inhibitory FcγRIIb were the highest on B cells (5.7×10^4). For FcγRIIIa on NK cells we calculated 2.2×10^5 binding sites whereas 4.4×10^4 (22) and 7.9×10^4 (35) receptor numbers were reported in other studies. Eosinophils had an ABC for FcγRIIIa of 8×10^3 binding sites according to our data compared to a receptor number of 1.2×10^4 reported by others (35). Our data on FcγRIIIa expression on non-classical monocytes revealed 2.3×10^5 sites per cell whereas numbers of



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median ABC [x10³ binding sites/cell]	FcγRI	FcγRIIa	FcγRIIb	FcγRIII
non-classical monocytes	6	84	4	230
classical monocytes	77	96	4	19
NK cells	<1	<1	2	217
B cells	<1	<1	57	<1
T cells	<1	<1	<1	1
neutrophils	1	160	2	1.4x10³
eosinophils	1	111	2	8

FIGURE 4 | Fc gamma receptors on human peripheral blood leukocytes. Depicted are box plots showing anti-FcR binding sites for **(A)** FcγRI, **(B)** FcγRIIa, **(C)** FcγRIIb, and **(D)** FcγRIIIa/b on indicated leukocyte subsets together with **(E)** a tabular presentation of the median number of binding sites. $n = 6$ (FcγRI, IIa, III) and 16 (FcγRIIb).

$\sim 3.5 \times 10^4$-1.2×10^5 have been published by others (18, 19, 22). For neutrophils we extrapolated an ABC for FcγRIII (i.e., mainly FcγRIIIb) of 1.4×10^6 binding sites per cell which is as well higher than the values provided by others (18, 19, 22–26, 34, 35) ($\sim 6 \times 10^4$-4×10^5) using antibody clones CLB-Gran/1, ION16, and mostly 3G8. As the anti-FcγRIII-PE fluorescence was beyond the fluorescence of the reference beads with respect to the highest number of antibody binding sites, the ABC values for neutrophils are being extrapolated by extending the calculated reference line (linear correlation in a log/log scheme) beyond the highest reference point. This reduces the accuracy of the calculation of anti-FcγRIII binding sites on human neutrophils, but in every experiment performed with human leukocytes so far the fluorescence of neutrophils upon anti-FcγRIII staining had

been higher than that of the respectively used reference beads with the highest binding capacity. This was also true in an assay where we used one lot of beads with ∼620,000 sites, the highest number of binding sites we noted so far.

Differential Expression of FcγRIIb on Human Monocytes

Upon quantification of FcγRIIb expression for a cohort of 10 healthy donors we found a pronounced variability in ABC values for FcγRIIb mainly on monocytes, which has also been observed by others (55, 56). Four of the donors revealed pronounced FcγRIIb expression on monocytes and were grouped as FcγRIIb^{+}, whereas the others had significantly lower FcγRIIb expression (FcγRIIb^{low}) (**Figure 5A**). Consistent with the published data, the variation was especially pronounced on CD16^{+} CD14^{low} non-classical monocytes. Whereas on classical monocytes the mean number of anti-FcγRIIb antibody binding sites was 1×10^4 for FcγRIIb^{+} donors vs. 3×10^3 for the FcγRIIb^{low} group, non-classical monocytes also revealed an ABC of 3×10^3 binding sites for FcγRIIb^{low} donors but even 1.7×10^4 for FcγRIIb^{+} donors (**Figure 5B**). Having identified the haplotypes of all donors regarding (i) the promoter polymorphism with either guanine at position −386 and thymine at position −120 or cytosine at −386 and adenine at −120 which affects transcription of the gene (57) and (ii) the transmembrane polymorphism of FcγRIIb which excludes the receptor from membrane rafts (58), we tried to correlate these haplotypes with the variances in FcγRIIb expression (**Figures 5C,D**). Among the 10 donors only two were heterozygous with respect to the promotor polymorphism carrying one allele of the−386C−120A haplotype whereas eight donors were homozygous "wild type" with the common−386G−120T (59). However, this promotor haplotype did not correlate with the observed dichotomy in expression on monocytes (**Figure 5C**). With respect to the FcγRIIb-232T variant, again no correlation with FcγRIIb expression level became apparent (58) (**Figure 5D**).

Since neither FcγRIIb haplotype accounted for the observed dichotomy in expression, we extended the analysis to known polymorphisms of FcγRIIa and IIIa. This includes the histidine vs. arginine polymorphism at position 131 of FcγRIIa (60) (referred to as position 133 in the original reference) as well as the valine vs. phenylalanine polymorphism at amino acid position 158 (or 176 if the leader sequence is included) of FcγRIIIa (61), which both affect IgG binding. We compared the respective high-affinity and low-affinity variants with the FcγRIIb expression (**Figures 5E,F**). The high affinity haplotypes for FcγRIIa carry at least one allele with a histidine at position 131 of FcγRIIa, whereas presence of two alleles encoding an arginine at this position represent the FcγRIIa low affinity haplotype (60). With respect to FcγRIIb expression, all three homozygous donors with 131R/R low affinity receptor (which, by chance, also carried the FcγRIIIa low affinity haplotype (see below)) revealed low expression of FcγRIIb on monocytes and heterogeneous expression on B cells. However, the high affinity haplotypes were heterogeneous for FcγRIIb expression on both monocytes and B cells (**Figure 5E**) (61). Whereas all donors with 158F/F low affinity receptors had rather low FcγRIIb expression, all those with pronounced expression of FcγRIIb carried the FcγRIIIa high affinity haplotypes (**Figure 5F**). For B cells we could not identify any single haplotype of those analyzed here or combinations thereof (not shown) that would correlate with the somewhat divergent expression of FcγRIIb on these cells (**Figures 5C–F**). Results on FcγRIIb expression from a second experiment performed for parallel quantification of activating and inhibitory receptors from six individuals (selected for containing two donors with FcγRIIIa 158 F/F, 158 V/F, and 158 V/V haplotype, respectively) were included in this data set. With the exception of a single sample with the homozygous FcγRIIIa high affinity haplotype but low FcγRIIb expression on monocytes—both datasets revealed corresponding results (**Supplementary Figure 8**). The individual data on age—which revealed no correlation with FcγRIIb^{low} and FcγRIIb^{+} phenotype—and FcγRIIa/FcγRIIIa haplotype from both experiments are depicted in **Supplementary Figures 9A,B**, respectively.

FcγR Expression on Basophils and Platelets

In addition to the major leukocyte populations in the peripheral blood we also quantified Fc receptor expression on murine and human basophils as well as human platelets (**Figure 6**). In full accordance with previous results (62) both murine and human basophils co-express activating and inhibitory Fcγ receptors. We verified that both human and murine basophils lack FcγRI expression (not shown). With 5.6×10^4 anti-FcγRIIb binding sites per cell, expression of the inhibitory receptor by murine basophils even exceeds that of B cells. This is contrasted by an even higher expression of the activating FcγRIII (7.1×10^4 binding sites per cell) whereas no FcγRIV expression was detected. Also human basophils revealed high FcγRIIb expression (9.5×10^4 binding sites). In contrast to murine basophils, expression of activating Fcγ receptors on human basophils is much smaller. As shown in **Figure 6B**, using the anti-FcγRII antibody IV.3 with pre-blocking FcγRIIb verified that the weak binding of IV.3 to basophils is not caused by low-affinity binding to FcγRIIb but indeed reflects moderate expression of FcγRIIa (8×10^3 binding sites per cell). With $\sim 4 \times 10^3$ anti-FcγRIII binding sites per cell, FcγRIII is expressed at very low levels. Of note, results of Meknache et al. suggest that this weakly expressed FcγRIII might in fact be GPI-anchored FcγRIIIb rather than signaling competent FcγRIIIa (54).

Finally, we studied human FcγR expression on platelets. In contrast to murine thrombocytes, human platelets are known to express Fcγ receptors, namely FcγRIIa. Our quantification of FcγRIIa on human platelets revealed low ABC values with $\sim 1 \times 10^3$ binding sites per cell. Relatively low expression of FcγRIIa by human platelets in comparison to the FcγR expressing leukocytes—has also been reported by others ($1.5–4.7 \times 10^3$ binding sites) (63–65).

Individual FcγR Expression Repertoires

Since for a cohort of six human donors the expression of FcγRI, FcγRIIa, FcγRIIb, and FcγRIII has been quantified in

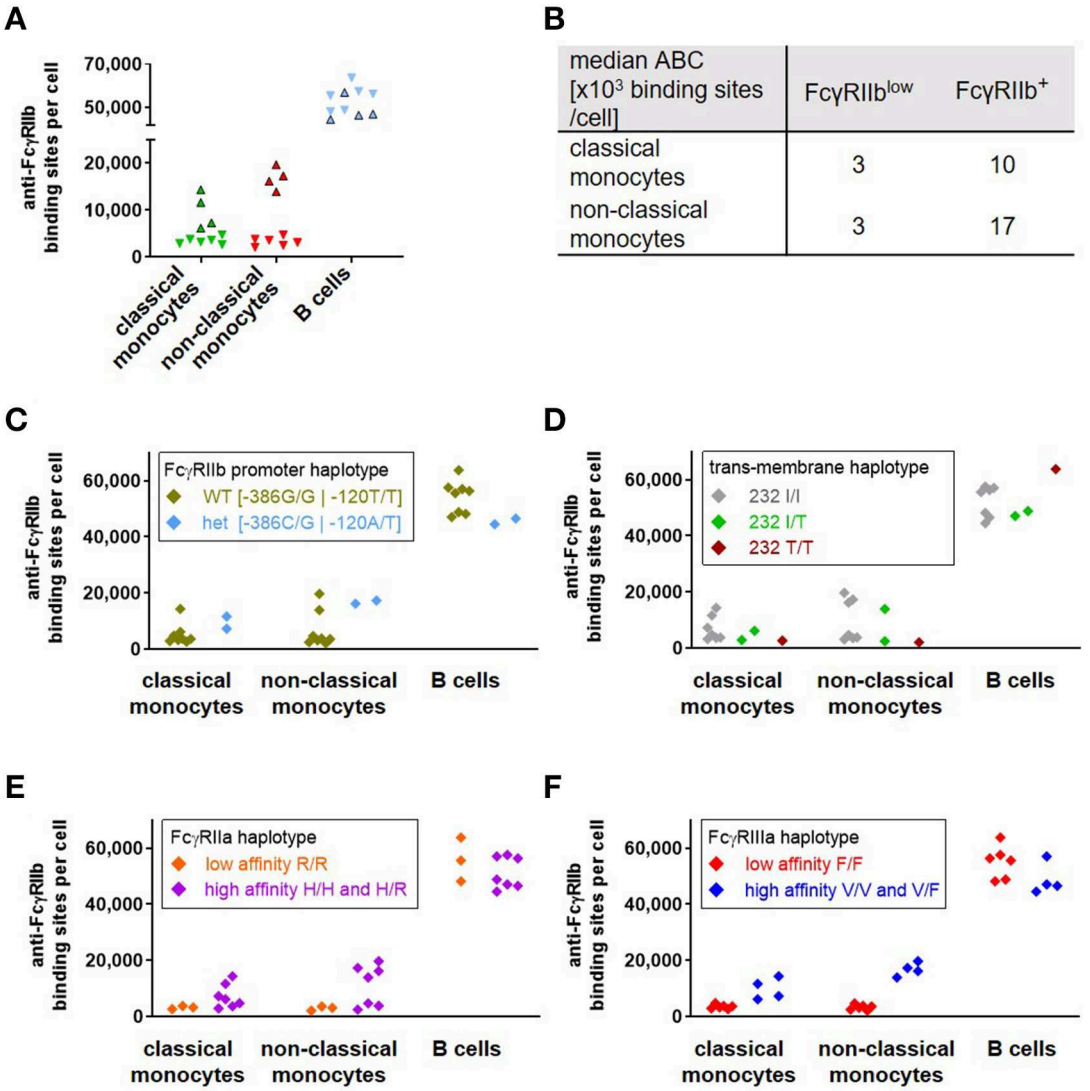

FIGURE 5 | Correlation of FcγRIIb expression with Fc receptor haplotypes in human donors. As depicted in **(A)**, a group of four of ten donors (FcγRIIb$^+$, bordered triangles) revealed a significantly higher expression of FcγRIIb (given as anti-FcγRIIb binding sites per cell) on monocytes than the others(FcγRIIb^{low}, inverted triangles without borders). Individual values are depicted for classical monocytes (green), non-classical monocytes (red) and B cells (blue). Median anti-FcγRIIb binding sites on monocyte subsets of FcγRIIb$^+$ and FcγRIIb^{low} groups are depicted in **(B)**. **(C–F)** Anti-FcγRIIb binding sites on monocytes and B cells of the ten donors are grouped according their respective haplotypes for **(C)** the coupled−386−120 promoter polymorphisms, **(D)** the isoleucine vs. threonine polymorphism at position 232 in the transmembrane region of FcγRIIb for homozygous I/I, T/T or heterozygous I/T donors, **(E)** the low affinity haplotype of FcγRIIa being homozygous with arginine at position 131 vs. the high affinity haplotype with at least one histidine allele and **(F)** the low affinity haplotype of FcγRIIIa, homozygous with phenylalanine at position 158 or the high affinity haplotype bearing at least one valine allele.

parallel, the respective expression pattern of the different Fcγ receptors can be assessed for each individual donor. Thus, for peripheral blood leukocytes effectively expressing more than one signaling-competent Fcγ receptor, i.e., monocytes, eosinophils, and basophils—both the relative fraction of its receptor repertoire as well as the absolute amount is shown in **Supplementary Figure 10** for each individual donor. These data show that in spite of individual differences the principal A_{rec}/I_{rec} with respect to predominance of either the activating Fcγ receptors (on monocytes and eosinophils) or the inhibitory receptor (on basophils) is conserved for the respective cell populations among the various donors.

DISCUSSION

The main purpose of our study was to quantify mouse and human FcγR expression on immune cells. From a qualitative point of view our results regarding the expression pattern of the different activating and the inhibitory FcγR on human and murine peripheral blood leukocytes correspond well with previously

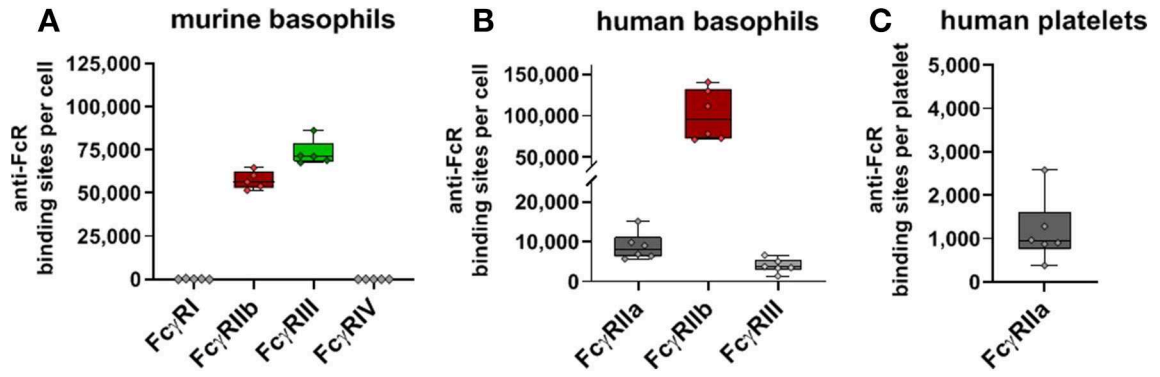

FIGURE 6 | Fc gamma receptors on basophils and platelets. Depicted are box plots showing anti-FcγR binding sites for receptors expressed on **(A)** murine or **(B)** human basophils, and **(C)** human platelets from five C57BL/6 mice or six human donors, respectively.

published data (1, 9–11, 66). However, the main purpose of the current study was to add a quantitative aspect to these relative expression values. Apart from allowing to assign numerical values for FcR expression for various cell types, this also allows to estimate ratios of activating and inhibitory receptors on a given cell population and e.g., quantitative comparison of receptor expression on murine and human leukocytes.

Experimental Caveats

The methodology used in this study to estimate and compare numbers of binding sites for antibodies for antigens of interest is better suited than qualitative statements on differences in fluorescence intensities. However, since the binding to the respective reference beads requires complete antibody molecules it should be noted that due to the bivalent nature of antibodies one anti-FcγR antibody may theoretically bind two receptor molecules if they are in close proximity and proper orientation to one another and, thus, numbers of antibody binding sites may under-represent the numbers of respective antigens as was observed for CD4 molecules on human leukocytes (37). However, experiments performed with the immobilized antigen indicated that bivalent binding takes place only if mobility of antigens in plasma membranes is sufficiently high. Thus, in spite of staining at low temperatures in our experiments to reduce membrane mobility, there is a theoretical possibility of systemic underrepresentation of the actual number of respective Fcγ receptor molecules by the ABC up to a factor of two at most. We, thus, strictly referred to ABC/anti-FcγR binding sites per cell in the context of this work. Antibodies could also bind to Fc receptors via their Fc portion, once the Fab binds with high affinity to its antigen. However, this could only affect the quantification (leading to an underestimation of the actual receptor numbers) if the Fc-binding receptor were the Fcγ receptor of interest in the respective measurement. In this case, the lower affinity binding via an Fc part competes with the specific high affinity binding via the Fab of the anti-FcγR detection antibody. Due to the anti-FcγR antibodies being present in saturating amounts in the experiments presented here, they will probably replace virtually all Fc domains bound to the respective receptor of interest and contribute normally to the total anti-FcγR-PE mediated fluorescence of the cell. Conversely, binding of anti-FcγR antibodies via their Fc part to other Fcγ receptors than the receptor of interest would represent unspecific binding and cause overestimation of actual receptor numbers. To avoid such unspecific Fc-mediated binding in the mouse system we used well-established blocking antibodies directed against Fc receptors that were not to be quantified. For experiments with human samples we used FcX™ Fc Receptor Blocking Solution which limits Fc-mediated binding to FcγRs by pre-occupying their Fc binding sites, but does not affect antigen-specific FcγR-detection. In physiologically active cells, pretreatment with FcγR binding antibodies can mediate receptor crosslinking, and, thus, induce signaling and changes in surface receptor numbers. Staining at low temperatures and in presence of sodium azide renders the cells physiologically inactive to avoid such potential influences of Fc block.

In most cases, where quantitative expression data of Fcγ receptors on distinct cell populations are available in the literature, these values are exceeded by the ABC values presented in this work. Besides the possibility that such differences may be inherent to the different methodologies being used for receptor quantification, conceivable reasons for the higher values presented here may be that (i) we use freshly isolated biological samples, and (ii) we aim at saturating conditions for anti-FcγR antibody binding to cells, which is critical for assays based on antibody-binding reference beads.

A/I Ratios of Murine Fc Receptors

Based on the different affinities of the various IgG isotypes to the activating and inhibitory Fc receptors the concept of the so called A/I ratio has been developed as a prediction of the outcome of binding of IgG molecules of a given isotype (13). The numerical data of the present work enable a corresponding concept for the ratio of activating (A_{rec}) and inhibitory receptors (I_{rec}) on immune cell subsets to better comprehend the roles of the distinct cell types in antibody-mediated effector functions. For example, based on our ABC values for the activating receptors FcγRI, FcγRIII and FcγRIV and inhibitory FcγRIIb, classical

monocytes have an overall A_{rec}/I_{rec} ratio of ∼2 with Fcγ receptor III providing a somewhat higher contribution to this A_{rec}/I_{rec} ratio than FcγRI. Taking into account the different affinities of these FcγRs to the various IgG isotypes, one may speculate that IgG1 or IgG2b may act mainly via FcγRIII and IgG2a may act via FcγRI. According to their respective A_{rec}/I_{rec} ratios either may be controlled by comparable amounts of FcγRIIb. According to our ABC values, non-classical murine monocytes have a total A_{rec}/I_{rec} ratio of ∼10 with an individual contribution of ∼1 by FcγRIII and ∼9 by FcγRIV. Thus, due to the very low affinity of IgG1 to FcγRIV—IgG1-mediated effects may be expected to induce signaling mainly via FcγRIII, whereas IgG2a and IgG2b responses should be dominated by FcγRIV. In models where non-classical monocytes play an important role, the lack of inhibitory receptors by using FcγRIIb knockout mice should affect mainly IgG1-mediated responses but have a much weaker effect on IgG2a and IgG2b responses, especially when taking into account (and, thus, implementing the classical A/I concept) the significantly higher affinity of IgG2a to FcγRIV compared to FcγRIIb. This may e.g., be one reason for earlier observations of isotype specific effects in mice lacking inhibitory Fcγ-receptor expression in anti-tumor responses (13, 67).

This concept of cell specific A_{rec}/I_{rec} ratios may be especially useful for a better understanding of effector functions mediated by subclasses with intermediate classical A/I ratio such as murine IgG2b as discussed in Nimmerjahn and Ravetch (13), or various murine or human glyco-variants, or by immune-complexes of mixed isotypes.

FcγR Knockout Mice

It is common practice to use knockout mice lacking one or several FcγRs to study the role of these receptors in IgG dependent immune responses. However, a compensatory upregulation of other Fcγ-receptors may complicate the interpretation of the results. In mice this may be of special relevance for activating Fc-receptors as all FcγRs and the high affinity FcεRI require the common FcRγ-chain for cell surface expression and signaling function (41, 68). Indeed, this seems to be the case for activating receptors FcγRIII and FcγRIV on cells which express both molecules (**Figures 2B,C**). As has been suggested earlier in analogy to increased FcγRIII expression on mast cells that lack the high affinity IgE receptor FcεRI (69) this may be due to the fact that the activating receptors compete for association with the common FcRγ-chain, which is essential for expression of the activating Fc receptor α-chains on the cell surface (51, 52). The relatively low expression of FcγRI (**Figure 1**), however, might be the reason why its knockout does not influence the expression of FcγRIV on non-classical monocytes or neutrophils (**Figure 2B**) or FcγRIII on any population (**Figure 2C**).

Functional Role Human and Murine NK Cells in ADCC

Both, human and murine NK cells, express only one activating receptor (FcγRIII in mice and FcγRIIIa in humans). However, in contrast to human NK cells, murine NK cells show the weakest expression of all tested FcγRIII-expressing leukocytes. This may have pronounced functional consequences: In humans, NK cells have been suggested to be key effector cells for antibody dependent cell mediated cytotoxicity (ADCC) (70). In contrast, due to their very low number of activating FcγRIII receptor molecules, a relevant role of NK cells in ADCC is unlikely in mice. This is in line with various experimental observations: (i) crosslinking of FcγRIII poorly activates murine NK cells in absence of additional stimulation (71); (ii) anti-CD20 mediated B cell depletion in mouse models is independent of NK cells but rather depends on FcγR expressing mononuclear phagocytic cells (72); (iii) anti-CD25 rat IgG1-mediated depletion of regulatory T cells is fully dependent on FcγRIII-expressing mononuclear phagocytic cells but is efficient in mice lacking NK cells (73, 74); (iv) the human anti-CCR4 monoclonal antibody KM2760 has anti-tumor activity both in murine and human experimental systems. However, whereas NK cell-mediated ADCC was suggested as the major anti-tumor effector mechanism in humans (75), in mice not NK cells but rather myeloid cells seem to be responsible for the anti-CCR4 dependent tumor cell depletion (75).

Variability in Human FcγRIIb Expression

Upon quantification of human FcγRIIb on leukocytes we found that one group of donors showed low ABC for FcγRIIb on monocytes whereas another group showed pronounced expression. This observation is consistent with published data (i) by Bruhns et al. showing that only a fraction of human donors revealed significant FcγRIIb expression on monocytes (55) and here especially on $CD16^{+}$ $CD14^{low}$ non-classical monocytes [referred to as patrolling monocytes in (55)] and (ii) by Glennie et al. where variable expression of FcγRIIb was found on non-classical $CD14^{lo}$ monocytes with lower expression on $CD14^{hi}$ monocytes (56). Since the dichotomy of FcγRIIb ABC values was so obvious and careful assessment of FcγRIIb in the blood of patients receiving a therapeutic mAb was suggested to be an important marker of prognostic value (56) we investigated whether this variation correlated to any FcγR-related factor. Among all four haplotypes, interestingly, only FcγRIIIa variants revealed a correlation with FcγRIIb expression on monocytes: The high-affinity FcγRIIIa was present in all individuals with pronounced FcγRIIb expression, whereas all individuals with the low affinity haplotype revealed low ABC for FcγRIIb on monocytes. More studies with larger cohorts of human donors will be necessary to verify and understand this correlation in more detail in the future. Regarding the frequency and linkage of FcγRIIa and FcγRIIIa our data from the first random cohort with 10 donors, corresponds to published data on Caucasian populations. In accordance e.g., with van der Pol et al. (76) the two allelic variants of FcγRIIa are equally represented (ten 131R alleles and ten 131H alleles) among the 20 FcγRIIa alleles present in this cohort of 10 donors, whereas the 158F variant of FcγRIIIa is more common than the 158V variant (14 158F alleles and six 158V alleles). In addition, a linkage disequilibrium between FcγRIIa and FcγRIIIa became apparent: For nine of the 10 donors the allelic combinations of FcγRIIa and FcγRIIIa can be unequivocally determined since they are homozygous for at least one allele. Among these 18 allelic combinations the

FcγRIIa^{131H} FcγRIIIa^{158V} (131H/158V) combination is present four times, the 131H/158F combination five times. 131R/158V and 131R/158F are present 1 and 8 times, respectively. This is in accordance with the enhanced co-occurrence of FcγRIIa^{131R} with FcγRIIIa^{158F} which has been described e.g., by Niederer et al. where a moderate/strong linkage disequilibrium between FcγRIIa and FcγRIIIa was found in a UK and Swedish Caucasian as well as a Kenyan cohort (77).

With respect to counterbalancing activating signals, it would be reasonable that cells with higher affinity activating Fcγ-receptor alleles would require a higher level of FcγRIIb expression to counter regulate this lower threshold for cell activation. Inversely, cells with low affinity activating receptor haplotypes require less pronounced counter regulation. This is in line with the low FcγRIIb expression on monocytes, consistently (but not exclusively) detectable in individuals with low affinity FcγRIIa/FcγRIIIa haplotypes. Following this line of argument, the modulation of inhibitory receptor expression by activating receptor haplotypes may well be cell type dependent, since this is relevant mainly for cell types that co-express activating and inhibitory receptors, such as monocytes. Accordingly, we found no obvious association of FcγRIIb expression with the activating receptor haplotypes. For the FcγRIIa$^{131H/R}$ haplotypes it was recently shown also by others that the corresponding SNP (SNP rs1801274) does not affect FcγRIIb expression on B cells (78).

How genetic regulation of FcγRIIb expression by activating receptor haplotypes might be achieved on a cellular and molecular level is not yet clear. However, one might envision that the association of FcγRIIb expression and the activating Fcγ-receptor affinity haplotype could be via a promoter/enhancer SNP that modulates expression of FcγRIIb in linkage disequilibrium with coding SNPs of activating Fc receptors as has been suggested by Roederer et al. (78).

FcγRIIIb Expression on Human Neutrophils

According to our results, FcγRIIIb seems to be expressed at exceedingly high levels by human neutrophils. This high amount of FcγRIIIb may be important to fulfill its biological function in immune-complex induced neutrophil activation. It has been suggested to be critical for tethering immune complexes to neutrophils (79). It may be a prerequisite for the proposed role of neutrophils to clear ICs under homeostatic conditions. This concept is supported by studies demonstrating low expression of FcγRIIIb to be associated with increased susceptibility to lupus nephritis and glomerulonephritis [reviewed in (80)]. Regarding neutrophil activation, a number of groups analyzed the function of the two receptors—FcγRIIa and FcγRIIIb expressed by neutrophils under steady state conditions [references in (81)]. Their tenor is that immune-complex induced activation of neutrophils requires both receptors. Since blocking of FcγRIIIb but not of FcγRIIa strongly affects immune-complex binding, FcγRIIIb is regarded as a kind of collector for immune complexes. However, the fact that FcγRIIIb is expressed in large excess compared to FcγRIIa (>10 fold according to our ABC values) raises the question by which stoichiometry and which receptor arrangement the co-engagement of both receptors with immune-complexes may take place. It should be noted that it has been reported very recently that neutrophils also carry low levels of FcγRIIIa, with this receptor being masked by the high levels of FcγRIIIb expression (82). Thus, a minor part of the anti-FcγRIII ABC values presented here may be derived from signaling-competent FcγRIIIa.

CONCLUSIONS

In summary, we present a comprehensive and comparative numerical quantification of Fcγ receptors on the main immune cell subsets in humans and the most common laboratory mouse strain C57BL/6J. These numerical data may enable improved stoichiometric considerations on A/I ratios regarding activating and inhibitory receptors as well as improved modeling of antibody-mediated immunological processes. In addition, we emphasize that conclusions drawn from knockout animal models have to be carefully evaluated in order to avoid potential misinterpretations due to compensatory modulation of other receptors.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Figshare repository: doi: 10.6084/m9.figshare.11604201.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Commission of the University of Erlangen-Nürnberg. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Government of Lower Franconia.

AUTHOR CONTRIBUTIONS

MB and CK performed experiments with equal contribution and analyzed data. MB and FN supervised experiments, interpreted data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00118/full#supplementary-material

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