THE B-SIDE OF B CELLS

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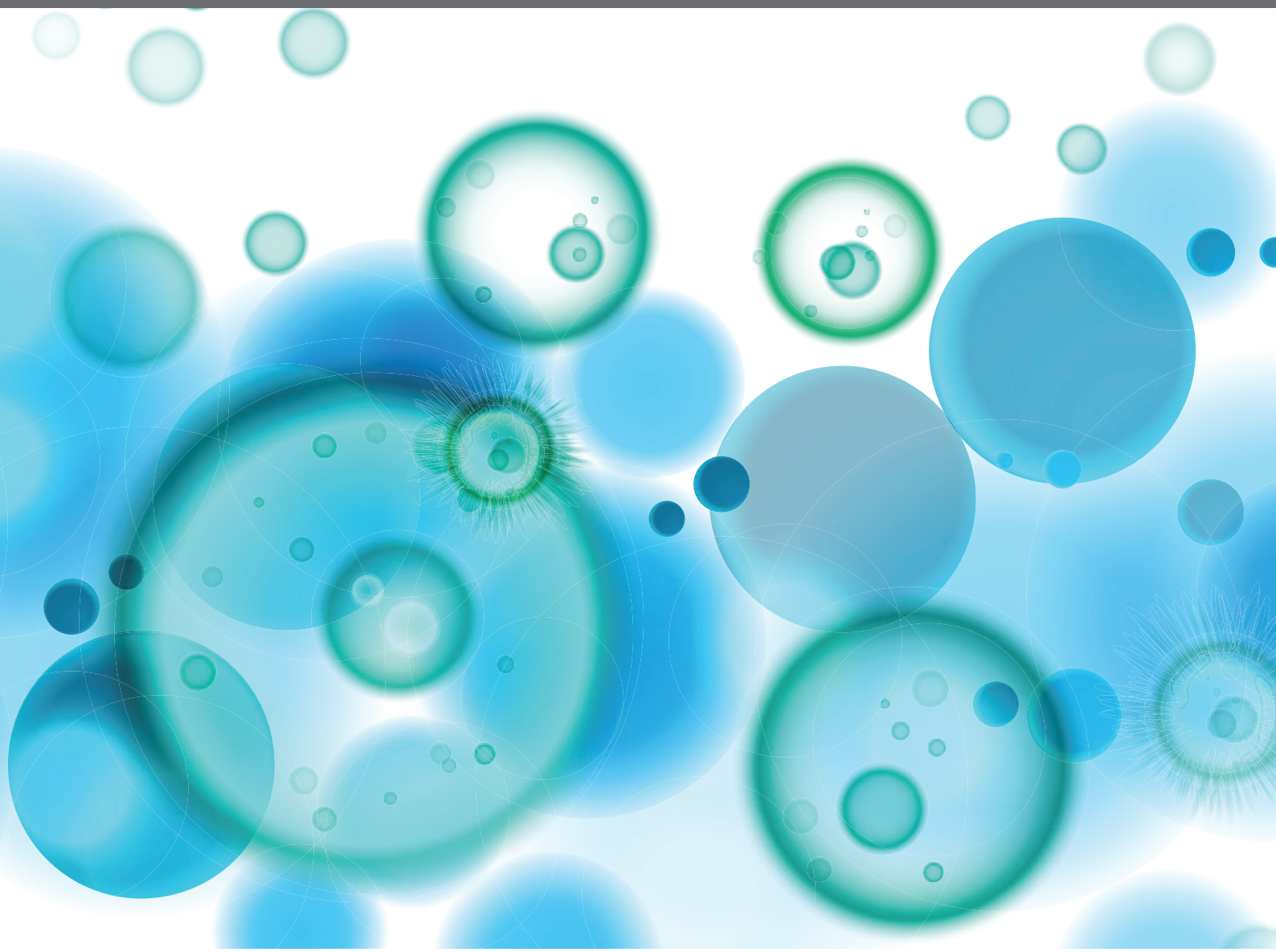

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THE B-SIDE OF B CELLS

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EDITORIAL
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Editorial: The B-Side of B Cells

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Keywords: B cells, IgM natural antibodies, marginal zone (MZ) B cells, IgA, tertiary lymphoid structure, bloodborne infections

Editorial on the Research Topic

The B-Side of B Cells

B cells play a pivotal role in the humoral immune response by secreting highly diverse but specific antibodies and recently many other B cell functions with important implications on the functional homeostasis of the immune system have been studied.

In this Research Topic we collected new data on the many non-conventional roles of B cells in health and in different disease settings.

Early B cell development and cell lineage commitment occurs in the fetal liver and continue in the bone marrow throughout life after birth. B cells can be subdivided into two main groups (1, 2). B1 cells produce polyreactive "natural" antibodies and are found primarily in the peritoneal and pleural cavities, while B-2 cells produce antigen-induced antibodies in secondary lymphoid organs. Immunoglobulin M (IgM) is the first antibody isotype expressed during development of both cell populations and forms the first humoral B cell receptor (BCR) on the surface of naive B cells. It has previously been assumed that induced IgM antibodies are short-lived and that the corresponding B cells class switch to more effective antibody isotypes. However, recent evidence suggests that IgM antibodies can be produce by long-lived plasma cells and possess memory phenotype with diverse antibody repertoire. How IgM structure and BCR signaling directs B cell development and their responses during infectious and non-communicable diseases is comprehensively reviewed in this issue by Jones et al. Deeper mechanistic insight into fetal B cell development and how it changes in postnatal life is essential for a detailed understanding of the role of B cells during the early onset and progression of disease.

B-cell acute lymphoblastic leukemia (B-ALL) is one of the most common cancers in children, with many of the leukemia-initiating events originating *in utero*. It is likely that the biology of B-ALL, including leukemia initiation, maintenance and progression depends on the developmental stage and type of B-lymphoid cell in which it originates. B-ALL is associated with multiple chromosomal translocations that often occur in the fetus and have been differentially associated with poor prognosis and response to treatment. Whether these differences are associated with their emergence in fetal progenitors which express genetic programs that favor self-renewal and proliferation has long been a matter of speculation and driven largely by observations in murine models. The advent of single cell sequencing technology has allowed the transcriptome of

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hematopoietic cells to be investigated in unprecedented detail. In the mini-review by Jackson et al. the central hypothesis of leukemogenesis is addressed in infant and fetal B cells leukemias which likely result from patterns of genes expressed at the corresponding stages of development. The authors draw their conclusions from recent advances in human fetal lymphoid and B cell progenitors, relevant to understand infant/childhood leukemia, as well as to settle emerging questions in the field (Jackson et al.).

Besides the well-known protective function against invading pathogens, B cells contribute to cellular immunity and regulate or enhance the immune response as antigen presenting cells or effector cells. Many studies have recently attempted to categorize B cells, similar to T cells, into "regulatory" or "effector", according to their cytokine production profile (3). The homeostasis between regulatory and effector compartments and crosstalk between different cell types are the conditions *sine qua non* for a functional and effective immune response. These multifaceted functions of B cells are currently deeply being investigated in health and diseases that include not only canonical immunological conditions, such as autoimmunity or immunodeficiencies, but also neoplastic, metabolic and neurologic disorders.

In the review by Willsmore et al. the authors discuss the role of B cells in tumor immunity focusing at the melanoma model and how the in deep characterization of B cell subset infiltrating the tumor or generating tertiary lymphoid structures (TLS) is, potentially, a tool to predict not only the response to immune checkpoint inhibitor (CPI) therapy but also tumor progression and metastasization. In fact, whereas infiltrates of regulatory B (Breg) cells may be an indicator of a negative prognosis, intra-tumoral TLS often leads to positive outcome. Generation of anti-tumor specific IgG1 immune responses in TLS potentiates anti-tumor antibody dependent cell-mediated cytotoxic (ADCC) reactions against the tumor by recruiting natural killer cells, cytotoxic T cells, monocytes/macrophages and neutrophils. Although the study of B cell biology in the tumor context is of extreme importance, the key issue that remains is the identification of the factors, present in the tumor microenvironment, that govern B cell fate and thus tumor persistence/survival.

During bacterial invasion the spleen, and particularly marginal zone (MZ) B cells, are key players in controlling blood borne disease by providing the initial round of antibodies. After a bloodborne bacterial infection, neutrophils promptly migrate to the MZ. Lo et al. demonstrated that MZ B cell-deficient mice are more susceptible to systemic *Staphylococcus aureus* infection than wildtype mice. In their article they showed that in the initial phase of infection MZ B cells are able to recruit neutrophils into the marginal zone area in an IL-6/CXCL1/CXCL2 dependent way. The generation of this intimate relation between neutrophils and MZ B cells is crucial for pathogen clearance (Lo et al.).

Interesting to note that same pathogens are able to subvert immune response by means not always clear. As described by Parihar et al., one example is that of *Mycobacterium tuberculosis* bacilli, where formation of tuberculous granulomas are induced, to which B cells actively participate, resulting in persistent infection. The noxious behavior of B cells, in this infection, is partially dependent on IL-4Rα signaling. In fact, mice caring IL-4Rα deficient B cells showed reduced lung pathology to *M. tuberculosis* triggered by the increase, in the lungs, of macrophage pro-inflammatory responses and killing effector functions, and by the local production *M. tuberculosis* specific neutralizing IgA antibodies (Parihar et al.). Translation of this observation into humans will be possible upon the study of B cells isolated from the lungs of infected people.

Hepatitis B virus (HBV) is a non-cytopathic virus, which means that liver damage occurs primarily through the host immune response. Knowledge of the role of B cells has focused primarily on their antibody secretion during the acute phases of the disease. However, recent evidence suggests that they also regulate the immune response beyond antibody secretion (Cai and Yin). The importance of B cells in the immunopathology of a chronic disease progression has long been ignored but has recently gained renewed interest. For example, treatment with the anti-CD20 monoclonal antibody (rituximab) is known to reactivate HBV replication, which can lead to hepatic relapses even in patients with cleared infection (4). In this issue, van Hees et al. investigated the extent to which B-cell activity is related to the different phase of hepatitis B (CHB) infection (5). They profiled the transcriptome of peripheral and intrahepatic B cells between the four clinical phases of CHB infection and healthy controls. Their results showed an important difference between the transcriptomes of intrahepatic and peripheral B cells during HBeAg seroconversion and active regulation of B cell signaling in the liver. The identified unique transcriptome signatures of peripheral and hepatic B cells provide a good resource for studying the microenvironment-dependent effects on B cell immune status.

The transmembrane activator and CAML interactor (TACI) is a receptor encoded by the gene *TNFRSF13B*, and it is crucial for B-cell differentiation and plasma cell survival. Mutations of this gene are often found in common variable immunodeficiency (CVID) and in IgA -deficiency. However, the vast majority of individuals with mutations in *TNFRSF13B* are healthy, with this gene being among the 5% most polymorphic genes in humans. In their review, Cascalho and Platt hypothesize that *TNFRSF13B* diversity might promote innate and adaptive B cell responses.

The role of the immune system in different metabolic disorders such as atherosclerosis and obesity is now emerging as a new field of research with interesting implication in the treatment strategy for these disorders. B cell immunity has been shown to have a particularly important role in atherosclerotic plaque formation and the group of Coleen A. McNamara significantly contributed to the advancement of knowledge in this field (6, 7). In this Research Topic her group shows that chemokine receptor 6 (CCR6) enhances B-1 cell number and IgM secretion in perivascular adipose tissue to provide atheroprotection in mice and demonstrates that in humans, expression of CCR6 on a putative B-1 cell population is significantly reduced in patients with a high degree of coronary artery disease, suggesting that B-1 cell-specific augmentation of CCR6 expression may be a potential therapeutic approach.

The Frasca and Blomberg group analyzes an interesting pro-inflammatory memory B cell subset, called double negative B cells, in patients with obesity and show that these cells are characterized by chronic immune activation, secrete autoantibodies and express the transcription factor T-bet (Frasca et al.). These cells resemble a B cell population found in aged female mice (aged B cells, ABCs) that seem to contribute to a generalized proinflammatory milieu characterizing basal inflammatory states associated with age (8).

Beyond the classical humoral immune responses, or the more familiar A-side, B cells play multiple roles, some of which have been discussed in this Research Topic. We believe that a deeper engagement in the study of the multifaceted functions of B cells as enhancers and regulators of immunity might shed light in the pathogenesis of, not only immune-mediated processes but also metabolic and tumoral diseases, optimizing B cell targeting therapies in different clinical settings.

AUTHOR CONTRIBUTIONS

MMR conceptualized the idea of the topic, wrote and revised the manuscript. AA, AC, SG and MV wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Kristiansen TA, Vanhee S, Yuan J. The Influence of Developmental Timing on B Cell Diversity. *Curr Opin Immunol* (2018) 51:7–13. doi: 10.1016/j.coi.2017.12.005
2. Kreslavsky T, Wong JB, Fischer M, Skok JA, Busslinger M. Control of B-1a Cell Development by Instructive BCR Signaling. *Curr Opin Immunol* (2018) 51:24–31. doi: 10.1016/j.coi.2018.01.001
3. Lund FE. Cytokine-Producing B Lymphocytes-Key Regulators of Immunity. *Curr Opin Immunol* (2008) 20(3):332–8. doi: 10.1016/j.coi.2008.03.003
4. Viganò M, Mangia G, Lampertico P. Management of Patients With Overt or Resolved Hepatitis B Virus Infection Undergoing Rituximab Therapy. *Expert Opin Biol Ther* (2014) 14(7):1019–31. doi: 10.1517/14712598.2014.912273
5. Van Hees S, Cuypers B, Bourgeois S, Groothuismink ZMA, Meysman P, Van der Vlies P, et al. Sorted B Cell Transcriptomes Point Towards Actively Regulated B Cell Responses During Ongoing Chronic Hepatitis B Infections. *Cell Immunol* (2021) 362:104283. doi: 10.1016/j.cellimm.2021.104283
6. Sage AP, Tsiantoulas D, Binder CJ, Mallat Z. The Role of B Cells in Atherosclerosis. *Nat Rev Cardiol* (2019) 16(3):180–96. doi: 10.1038/s41569-018-0106-9
7. Srikakulapu P, McNamara CA. B Cells and Atherosclerosis. *Am J Physiol Heart Circ Physiol* (2017) 312(5):H1060–7. doi: 10.1152/ajpheart.00859.2016
8. Cancro MP. Age-Associated B Cells. *Annu Rev Immunol* (2020) 38:315–40. doi: 10.1146/annurev-immunol-092419-031130

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Therapeutic Cytokine Inhibition Modulates Activation and Homing Receptors of Peripheral Memory B Cell Subsets in Rheumatoid Arthritis Patients

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Memory B cells have known to play an important role in the pathogenesis of rheumatoid arthritis (RA). With the emergence of B cell-targeted therapies, the modulation of memory B cells appears to be a key therapeutic target. Human peripheral memory B cells can be distinguished based on the phenotypic expression of CD27 and IgD, characterizing the three major B cell subpopulations: CD27+IgD+ pre-switch, CD27+IgD- post-switch, and CD27-IgD- double-negative memory B cells. We evaluated different memory cell populations for activation markers (CD95 and Ki-67) and chemokine receptors (CXCR3 and 4) expressing B cells in active RA, as well as under IL6-R blockade by tocilizumab (TCZ) and TNF-α blockade by adalimumab (ADA). Memory B cells were phenotypically analyzed from RA patients at baseline, week 12, and week 24 under TCZ or ADA treatment, respectively. Using flow cytometry, surface expression of CD95, intracellular Ki-67, and surface expressions of CXCR3 and CXCR4 were determined. Compared with healthy donors (n = 40), the phenotypic analysis of RA patients (n = 80) demonstrated that all three types of memory B cells were activated in RA patients. Surface and intracellular staining of B cells showed a significantly higher percentage of CD95+ ($p < 0.0001$) and Ki-67+ ($p < 0.0001$) cells, with numerically altered CXCR3+ and CXCR4+ cells in RA. CD95 and Ki-67 expressions were highest in post-switch memory B cells, whereas CD19+CXCR3+ and CD19+CXCR4+ expressing cells were substantially higher in the pre-switch compartment. In all subsets of the memory B cells, *in vivo* IL-6R, and TNF-α blockade significantly reduced the enhanced expressions of CD95 and Ki-67. Based on our findings, we conclude that the three major peripheral memory B cell populations, pre-, post-switch, and double-negative B cells, are activated in RA, demonstrating enhanced CD95 and Ki-67 expressions, and varied expression of CXCR3 and CXCR4 chemokine receptors when compared with healthy individuals. This activation can be efficaciously modulated under cytokine inhibition *in vivo*.

Keywords: B cells, inflammation, adalimumab, tocilizumab (IL-6 inhibitor), memory B cells, rheumatoid arhritis

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory systemic autoimmune disease characterized by polyarthritis with swelling, pain, inflammation, and progressing destruction of joints. It affects approximately 1% of the population worldwide. RA causes considerable morbidity, diminishes the life quality, and increases mortality with increasing age (1, 2). RA is influenced by environmental and genetic factors, with obesity, diet, smoking, and microbial infections are known to induce RA in genetically susceptible individuals (3, 4). Inflammation of joints is the hallmark of RA, comprising a syndrome of pain, stiffness, and symmetrical synovitis of diarthrodial joints. Furthermore, systemic inflammation targets other organs, with substantial comorbidities observed in neurological, cardiovascular, and metabolic systems in this inflammatory joint disease (5, 6).

The key role of B cells has been appreciated since the discovery of the rheumatoid factor (RF). Along with anti-cyclic-citrullinated peptide autoantibodies, the RF serves as a disease, as well as severity biomarker (7). Patients with RA show a heterogeneous modulation of the B cell compartment, particularly with an increased frequency of memory B cells (8, 9). Enhanced B cell activity has been proposed in the pathogenesis of RA, along with different pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) critically involved in chronic inflammation. With a growing understanding of mechanistic pathways regarding B cell involvement in self-destruction during autoimmune diseases, there is strong evidence suggesting that B cells play a central role in the pathogenesis of several autoimmune diseases (10–12). Reportedly, a murine model of multiple sclerosis has reported that IL-6 producing B cells enhance T cell stimulation, including IL-17 polarization (13). IL-6 is a frontier cytokine in the induction of inflammation and generation of acute phase reactions, as well as regulation of immune responses. It is a multifunctional cytokine acting as a stimulator of B cells and was formerly described as a late-acting B cell differentiation factor for antibody-forming cells and germinal center reactions. Overproduction of IL-6 has been reported in several inflammatory autoimmune diseases, including RA, systemic lupus erythematosus, and systemic juvenile idiopathic arthritis (14, 15). Elevated IL-6 levels have also been linked with disease activity and progression in RA (16). TNF- α is a key pathogenic cytokine, playing a central role in RA through the activation of a cytokine cascade driving the inflammation and tissue damage. TNF-α is produced by various cell types, including lymphocytes (B and T cells), monocytes, macrophages, dendritic cells, synovial fibroblasts, mast cells, and natural killer cells (17–19). Along with IL-1β, TNF-α up-regulates RANKL (receptor activator of nuclear factor kappa-B ligand) and promotes osteoclast differentiation and bone resorption (20, 21). Serum levels of TNF-α have been shown to negatively correlate with B cell functions. Furthermore, an increased plasma level of TNF-α can induce TNF-α production from unstimulated B cells without antigenic stimulation, resulting in B cells possessing a pre-activated phenotype which renders them incapable of normal functions (22, 23).

Biological agents targeting key pro-inflammatory cytokines, including IL-6 (24, 25), and TNF-α, have substantially advanced in the treatment of autoimmune disorders (26). Tocilizumab (TCZ) is a humanized recombinant IgG1 monoclonal antibody that binds to the soluble and membrane-bound IL-6 receptor (27, 28). Adalimumab (ADA) is a fully human monoclonal antibody binding TNF, approved for the treatment of RA, either alone or in combination with disease-modifying antirheumatic drugs (DMARDs) demonstrating substantial experience in terms of efficacy and safety (29).

Enhanced B cell activity, particularly memory B cells, has drawn interest in evaluating the therapeutic response to biologics. B cell activation and its modulation through cytokine inhibition therapies have not been thoroughly investigated. Therefore, in this study, we aimed to explore the impact of distinct cytokine inhibitions on the activation status of B cell subpopulations. Hence, we analyzed the activation status and chemokine receptors expressed in different B cell compartments in RA during *in vivo* IL-6R (tocilizumab) and TNF-α (adalimumab) inhibition. Our results suggested that chronic inflammation leads to changes in chemokine receptor expression on peripheral blood B cells. This activation can be successfully modulated using cytokine inhibition therapies.

MATERIALS AND METHODS

Patients

In total, 80 patients with RA, with a median age of 53 years (range 35–73 years), and 40 age-matched healthy donors (HD) were selected in this study. These patients presented with active RA and were regarded as inadequate responders to classical treatment with conventional synthetic DMARD (csDMARD). These patients demonstrated a median disease duration of 8 years (range 2–28), and 73% were female. Patients were considered eligible for study participation if they met the revised 1987 criteria of the American College of Rheumatology (ACR) for RA classification or the 2010 ACR/European League Against Rheumatism (EULAR) classification criteria (30). The study protocol was approved by the ethics committee of the University Hospital, Würzburg, Germany, and was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients. Human material was stored according to standards of the Interdisciplinary Bank of Biomaterials and Data Würzburg at the University of Würzburg (see: www.ibdw.uk-wuerzburg.de). The enrolled patients (n = 60) were administered 8 mg/kg TCZ every 4 weeks as a 60-min infusion in combination with methotrexate (MTX). In parallel, for TNF-α inhibition, 20 patients were administered ADA at a dose of 40 mg every 2 weeks in combination with MTX. The primary endpoint was set at 12 weeks, with an extension period to 24 weeks for both TCZ and ADA.

Clinical Assessments and Evaluation of Efficacy

Demographic and clinical characteristics of patients were regularly monitored by measuring Disease Activity Score 28

(DAS28), RF levels, C-reactive protein (CRP) levels, and erythrocyte sedimentation rate (ESR) values. Before therapy, DAS28 scores for TCZ treated patients were 5.16 ± 1.31 (mean ± SD) with 95% CI (4.75–5.79) and of ADA treated patients were 4.78 ± 0.9 (mean ± SD) with 95% CI (3.99–5.57) before therapy. Furthermore, CRP levels were similar in TCZ treated (0.59 ± 0.09 mg/dl) and ADA treated (0.88 ± 0.4 mg/dl) patients before therapy. **Table 1** summarizes the clinical characteristics of patients receiving TCZ and ADA therapy. During treatment, DAS28 declined significantly at week 12 and week 24 ($p < 0.0001$), respectively. After the first infusion, inflammatory parameters, CRP and ESR, declined significantly, and stayed negative throughout the subsequent study period. No serious adverse events or serious infections were observed during the study. Similar effects were observed in patients treated with either TCZ or ADA.

TABLE 1 | Patients characteristics and clinical evaluation of effectiveness.

	Baseline	Week 12	Week 24
Age, Median (range) years	53 (35–73)		
% of female	73%		
Disease duration, mean (range) years	8 (2–28)		
RF positive	59		
ACPA positive	55		
RF positive /ACPA positive	55		
RF positive/ACPA negative	11		
RF negative/ ACPA negative	14		
Concomitant DMARD (patients)	60 TCZ, 20 ADA		
Concomitant Steroid (patients)	36 TCZ, 11 ADA		
Number of Previous DMARD	3 (Methotrexate, Leflunomide, Azathioprine)		
Patients with Rituximab therapy	0		
Patients completed the TCZ study	60	58	53
Patients completed the ADA study	20	20	19
Tocilizumab (*n* = 60)	***n* = 60**	***n* = 58**	***n* = 53**
DAS28 score (mean ± SD)	5.16 ± 1.31	3.22 ± 1.31ψ	2.65 ± 1.22ψ
CRP, mg/dl (mean ± SEM)	0.59 ± 0.09	0.14 ± 0.05ψ	0.08 ± 0.03ψ
ESR, mm/hour (mean ± SEM)	29.41 ± 3.15	9.06 ± 1.30ψ	7.11 ± 0.84 ψ
Patient's VAS. (mean ± SEM)	58.21 ± 4.16	46.97 ± 4.21 ψ	33.21 ± 4.74 ψ
Adalimumab (*n* = 20)	***n* = 20**	***n* = 20**	***n* = 19**
DAS28 score (mean ± SD)	4.78 ± 0.9	2.43 ± 0.71 ψ	1.91 ± 0.61 ψ
CRP, mg/dl (mean ± SEM)	0.88 ± 0.4	0.25 ± 0.05ψ	0.12 ± 0.04 ψ
ESR, mm/hour (mean ± SEM)	26.67 ± 6.1	12.86 ± 3.3ψ	8.57 ± 1.7 ψ
Patient's VAS (mean ± SEM)	57.14 ± 7.3	26.43 ± 7.5 ψ	19.29 ± 2.9 ψ

The primary endpoint of the study was a reduction in the Disease Activity Score in 28 joints (DAS28) at week 24. Except where indicated, values are the mean ± SEM. RF, rheumatoid factor; ACPA, anti-citrullinated peptide/protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; and VAS, visual analog scale (100-mm). $^{\psi}$ *P < 0.05 vs baseline.*

Flow Cytometry

EDTA anticoagulated peripheral blood was used for phenotype analysis by flow cytometry. In detail, 200 μL of whole blood was lysed in 2 mL of VersaLyse at room temperature for 15 min. Then, cells were washed twice with FACS buffer, followed by resuspension in the appropriate antibody preparation and incubated for 20 min at 4°C. For intracellular staining of Ki-67 expression, we used permeabilization and fixation method using eBioscience perm/fix kit (ebioscience cat no. 88-8824-00). The following monoclonal antihuman antibodies were used in appropriate concentrations to stain cells: anti-CD45-Krome orange (Beckman Coulter, cat no. 96416), anti-CD14-PC5.5 (Beckman Coulter, cat no. A70204), anti-CD 19-APC-Alexa fluor 750 (Beckman Coulter, cat no.A94681), anti-CD27-PE (BD Pharmingen, cat no.555441), anti-CXCR3-APC (BD Pharmingen, cat no. 561732), anti-CXCR4-APC (BD Pharmingen, cat no. 560936), anti-CD95-APC (BD Pharmingen, cat no. 558814), anti-ki-67-PE (Ebioscience Cat no. 12-5699-42), and anti- IgD-FITC (BD Pharmingen, cat no. 555778). After staining, the cells were analyzed by 10-color flow cytometry (Navios, Beckman Coulter), with at least 20,000 CD19+ events collected for each analysis.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, United States) and SPSS version 22 (IBM Corp., Armonk, NY, United States). The values were compared with baseline levels using the Mann–Whitney *U* test and the nonparametric Wilcoxon matched-pair test. Univariate logistic regression was performed to calculate the odds ratios and correlated using Pearson's *r*. All *p*-values ≤ 0.05 were considered statically significant. $^{***}p < 0.0001$, $^{**}p < 0.001$, and $^{*}p < 0.01$.

RESULTS

High Prevalence of Activated B Cells in RA Patients

To evaluate the activation status and homeostatic proliferation of B cells during active RA, we analyzed the surface expression of CD95 and intracellular Ki-67 expression on B cell subsets. Patients with RA demonstrated a significantly high number of both CD19+CD95+ and CD19+Ki-67+ B cells when compared with HD (**Figure 1** and **Supplementary Figure 1**).

In patients with RA, the mean frequency of CD19+CD95+ on B cells was 12.7 ± 0.6% (mean ± SEM) when compared with 6.3 ± 0.4% in HD ($p < 0.0001$). Based on the surface expression of IgD and CD27, human peripheral CD19+B cells can be divided into four subsets (31): mature naïve B cells (CD19+IgD+CD27-), pre-switch (CD19+IgD+CD27+),

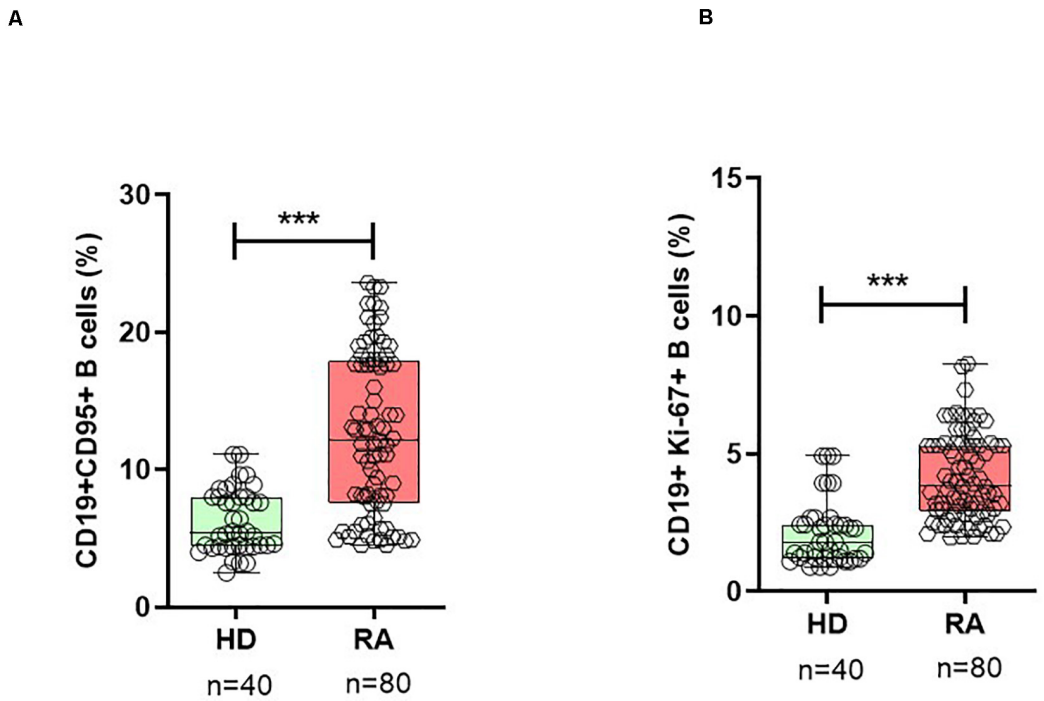

FIGURE 1 | Activated B cells are expanded in RA. In patients with RA patients, B cells show a significantly higher percentage of surface CD95+ **(A)** and intracellular Ki-67+ **(B)** expressing B cells when compared with healthy donors (HD). Data are shown in box-whisker plots showing individual dots, where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. *P* values were determined with the Student's unpaired *t*-test using GraphPad Prism 6 (***$P < 0.0001$). *n* = number of individuals.

post-switch (CD19+IgD-CD27+) conventional, and double-negative (DN; CD19+IgD-CD27-) largely "atypical" memory B cells (**Supplementary Figure 1**). Analysis of B cell subsets for CD19+CD95+ expressing cells showed that the post-switch subset presented the highest expression of CD95+ cells, followed by DN and pre-switch memory B cells, respectively, in RA patients (**Figure 2**). Compared with all three memory B cell subsets, the naïve B cell subset demonstrated significantly low CD95 surface expression (**Figure 2** and **Supplementary Figure 2**). In detail, the mean frequency of CD19+CD95+ expressing cells was as follows: in post-switch memory (CD19+IgD-CD27+CD95+) cells ($p < 0.0001$, mean ± SEM in RA = 38.9 ± 1.5% vs. HD = 18.6 ± 1.4%); in pre-switch memory (CD19+IgD+CD27+CD95+) B cells ($p < 0.0001$, RA = 19.7 ± 1.2% vs. HD = 9.2 ± 0.96%); in DN B cells ($p < 0.0001$, RA = 22.1 ± 1.0% vs. HD = 12.0 ± 0.9%); and in naïve B (CD19+IgD+CD27-CD95+) cells ($p = 0.018$, mean ± SEM in RA = 1.3 ± 0.1% vs. HD = 0.86 ± 0.1%).

The analysis of intracellular Ki-67 expression on B cells (CD19+Ki-67+) demonstrated that patients with RA presented significantly higher expression (4.2 ± 0.2) when compared with HD (2.2 ± 0.2; $p < 0.0001$) in HD (**Figure 1B**). Ki-67 expressing cells were significantly higher in all three memory B cell subsets, except in naïve B cells. The highest Ki-67 expression was observed in post-switch memory (mean 7.8%) B cells (CD19+IgD-CD27+Ki-67+), followed by pre-switch memory (mean 5.3%) B cells, and DN memory B cells (mean 3.8%), respectively, (**Figure 2C**). The expression of this marker was significantly higher in RA patients when compared with HD. In detail, post-switch memory B cells ($p = 0.013$, mean ± SEM in RA = 7.8 ± 0.6% vs. HD = 4.9 ± 0.7%); pre-switch memory (CD19+IgD+CD27+Ki-67+) B cells ($p = 0.0002$, RA = 5.3 ± 0.5% vs. HD = 2.2 ± 0.3%); DN memory B (CD19+IgD-CD27-Ki-67+) cells ($p = 0.010$, RA = 3.8 ± 0.3% vs. HD = 2.5 ± 0.3%), and in naïve B (CD19+IgD+CD27-Ki-67+) cells ($p = 0.89$, RA = 0.6 ± 0.1% vs. HD = 0.6 ± 0.1%).

Activated B Cells Positively Correlate With Disease Activity

As activated B cells might reflect disease activity, we correlated CD95+ and Ki-67+ expressing B cells in RA patients. Interestingly, using linear Pearson's correlation, we observed a positive correlation between the DAS28 score and CD95 expression ($r^2 = 0.35$, $p = 0.0001$). Similarly, DAS28 was significantly correlated with Ki-67 expression ($r^2 = 0.35$, $p = 0.0001$; **Figure 3A**).

As the subset compositions of the B cell compartment may be influenced by disease activity, we assessed the potential correlation between DAS28 and the percentage of each B cell subset. Herein, we observed a positive correlation between DAS28 (**Figure 3B**) and post-switch memory B cells ($r^2 = 0.097$, $p = 0.049$). However, pre-switch memory B cells were negatively correlated ($r^2 = 0.197$, $p = 0.02$), with no correlation observed

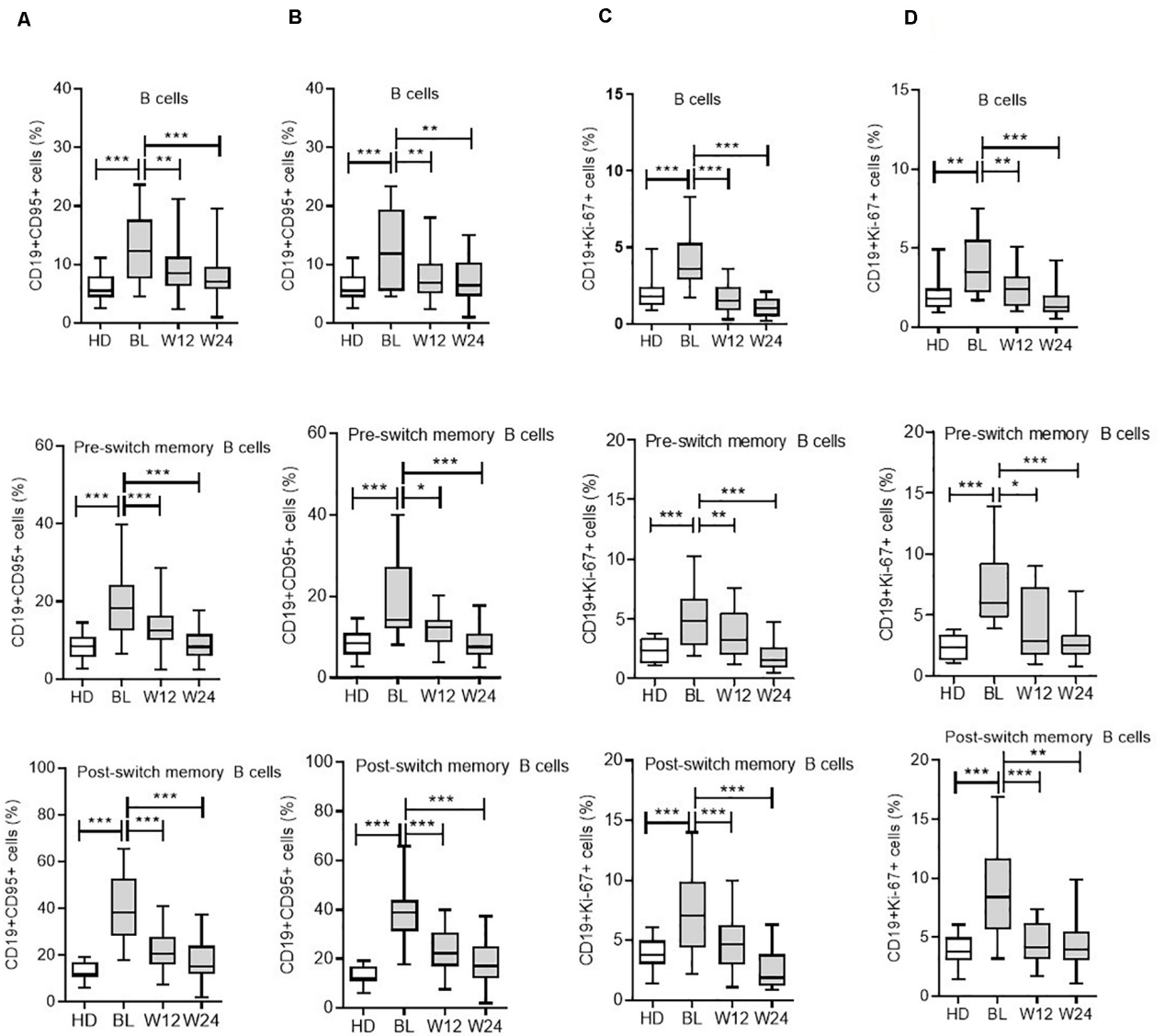

FIGURE 2 | Modulation of CD95 expression on B cell subsets during IL-6R and TNF-α inhibition therapy. For CD19+CD95+ expressing cells from all B cell subsets, including total B cells, pre-switch memory, and post-switch memory B cells, are significantly reduced during IL-6R inhibition **(A)** and TNF-α inhibition **(B)** therapy. CD19+Ki-67+ expressing cells from all B cell subsets are significantly reduced during IL-6R inhibition **(C)** and TNF-α inhibition **(D)** therapy. Values were consistently compared with baseline levels by using the Student unpaired *t*-test (***$p < 0.0001$, **$p < 0.001$, and *$p < 0.01$). BL = baseline, W12 = week 12, and W24 = week 24.

between DAS28 and DN B cells ($r^2 = 0.001$, $p = 0.879$). During treatment, non-significant correlation was observed at W12 and W24 (**Supplementary Figure 3**).

Modulation of CD95 and Ki-67 Expressions on B Cells Following IL6 Receptor Inhibition Induced by Tocilizumab

Subsequently, we determined whether therapy using an anti-IL-6R inhibitor (TCZ) can modulate CD95+ and Ki-67+ expressing B cells, as well as their subsets. Interestingly, both CD95 and Ki-67 expressions were significantly reduced in the B cell compartment during cytokine inhibition with TCZ (**Figure 2C** and **Supplementary Figure 2**). In detail, during TCZ therapy, CD95+ expressing B cells were significantly reduced from 12.5 ± 0.7% (mean ± SEM) to 9.5 ± 0.7% at week 12 ($p = 0.007$), and further reduced to 8.0 ± 0.6% at week 24 ($p < 0.0001$). Additionally, an analysis of B cell subsets demonstrated a significant reduction in CD19+CD95+ expressing cells in all B cell subsets investigated (**Figure 2A**).

Similarly, Ki-67+ expressing B cells were significantly reduced from 4.1 ± 0.3% (mean ± SEM) to 1.7 ± 0.2% at week 12 ($p < 0.0001$) and 1.0 ± 0.1% at week 24 (<0.0001). This reduction in CD19+Ki-67+ cells following IL-6R inhibition was observed across all B cell subsets (**Figure 2C**).

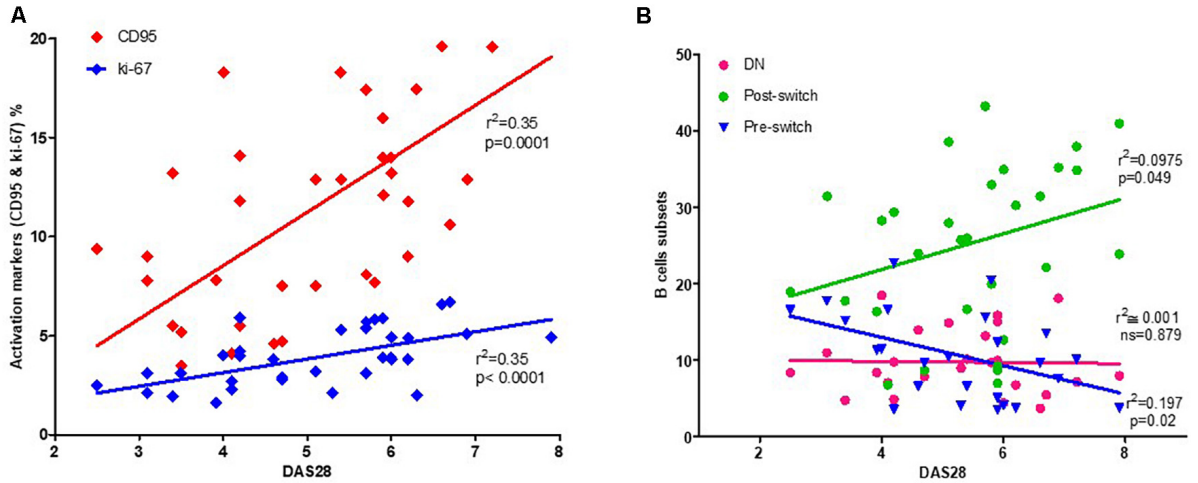

FIGURE 3 | Correlation between activated B cells and disease activity. A significant correlation can be observed between activated B cells, identified by their CD95+ or Ki-67+ expression, and DAS28 as a measure of RA disease activity **(A)**. Among B cell subsets, post-switch exhibits a positive correlation, pre-switch a negative correlation, while double-negative (DN) B cells fail to demonstrate any correlation with DAS28 **(B)**. The relationship between variables was evaluated using the Pearson linear correlation test. A two-sided *p*-value of <0.05 was considered statistically significant.

Modulation of CD95 and Ki-67 Expression by B Cells During TNF-α Blockade

To compare the effects of different cytokine inhibition therapies on the activation state of B cells, we analyzed CD19+CD95+ and CD19+Ki-67+ expressing cells during TNF-α blockade using ADA. Similar to TCZ, following ADA therapy, CD95 expression on B cells was significantly reduced (**Figure 2B**) from 13.1 ± 1.6% (mean ± SEM) to 7.8 ± 0.8% at week 12 ($p = 0.004$) and 7.5 ± 0.8% at week 24 ($p = 0.002$). Furthermore, the B cell subset analysis presented corresponding reduced CD95+ cells (**Figure 2B**).

During ADA treatment, analysis of CD19+Ki-67+ expressing B cells revealed reduced expression of Ki-67, from 4.0 ± 0.5% (mean ± SEM) to 2.5 ± 0.3% at week 12 ($p = 0.02$) and 1.5 ± 0.3% at week 24 ($p < 0.0001$). Likewise, the intracellular expression of Ki-67+ cells, an indicator of proliferation, was significantly reduced in all B cell subsets during ADA treatment (**Figure 2D**). These findings indicate that ADA can modulate the activation status of B cells in RA.

Chemokine Receptor CXCR3 and 4 Expressions by B Cells and Their Modulation Using Biologics

Under pathological conditions, chemokines are known to direct lymphocytes into inflamed tissues by interacting with chemokine receptors. Therefore, we questioned if, and how, the expression of chemokine receptors, CXCR3, and CXCR4, on B cells is affected in patients with active RA. In both CD19+CXCR3+ and CD19+CXCR4+ chemokine receptors expressing B cells, we observed a non-significant numerical increase in RA patients when compared with HD (**Figure 4**). In detail, CD19+ CXCR3+ expressing B cells comprised 53.3 ± 2.9% (mean ± SEM) in RA and 47.1 ± 3.7 % in HD ($p = 0.190$), whereas the CD19+CXCR4+ B cells were 66.3 ± 2.5% in RA and 61.3 ± 3.4% in HD ($p = 0.24$). Among the different B cell subsets, naïve B (CD19+IgD+CD27-) cells numerically presented the highest percentage of CD19+CXCR3+, as well as CD19+CXCR4+ cells, followed by pre-switch memory B cells, DN B cells, and post-switch memory B cells, respectively.

Interestingly, we observed significant alternations in CXCR3 chemokine receptors expressing B cells during cytokine inhibition therapies. In detail, during TCZ treatment, the mean CD19+CXCR3+ expressing cells increased from a baseline value of 52.3 ± 3.1 to 63.2 ± 2.7% ($p = 0.010$) at week 12 and 65.3 ± 3.6% ($p = 0.011$) at week 24. A similar pattern was observed in all B cell subsets during TCZ treatment (**Figure 5A** and **Supplementary Figure 4**). Similarly, during ADA treatment, the expression pattern of CD19+CXCR3+ B cells reduced from 68.2 ± 4.6% at baseline to 54.2 ± 3.3% at week 12 ($p = 0.019$), with subsequent elevation to 72.2 ± 3.1% ($p = 0.47$) at week 24 for total B cells. Similar significant changes were observed in the expression pattern of CD19+CXCR3+ cells at week 12; these changes were non-significant at week 24 for all B cell subsets (**Figure 5B**).

Contrary to CXCR3 modulation during cytokine inhibition therapies, CD19+CXCR4+ receptor expressing B cells presented a reduced or unchanged pattern following TCZ or ADA treatment. Among B cell subsets, the CD19+CXCR4+ expressing cells were significantly decreased in post-switch memory B cells during both treatments, with a significant decrease observed in DN B cells after ADA treatment at week 12. However, these differences were mild at week 24 (**Figures 5C,D** and **Supplementary Figure 4**). These findings suggested that the CD19+CXCR3+ and CD19+CXCR4+ expressing B cells present distinct patterns during active RA disease, which were differentially modulated during cytokine inhibition therapies.

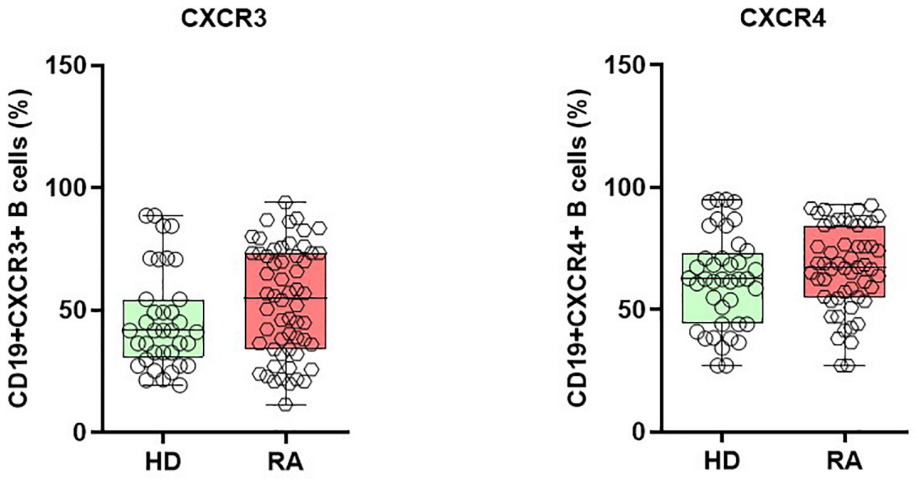

FIGURE 4 | Enhanced expression of chemokine receptor CXCR3 and CXCR4 on B cells during active RA. CD19+CXCR3+ and CD19+CXCR4+ expressing B cells are elevated in patients with active RA when compared with healthy donors (HD).

DISCUSSION

The use of monoclonal antibodies against cytokines has opened new therapeutic modalities for patients with RA. Currently, specific biologics such as TNF-α inhibitors and IL-6 receptor antibodies are considered highly efficacious therapeutic agents in RA treatment. Immune cell monitoring may help assess these therapies to reach optimal responses (3, 32, 33). Therefore, the main objective of this study was to explore the impact of *in vivo* IL-6 or TNF-α inhibition on the activation status of B cells. Hence, we analyzed activation status and chemokine receptor expressions in different B cell subsets from RA patients during IL-6R (TCZ) and TNF-α (ADA) inhibition. Previous data from experimental arthritis have shown a gender-based differences in B cell signatures (34, 35), however, in our cohort, we did not observe any gender-biased variation in B cell subsets (data not shown). We longitudinally analyzed patients undergoing treatment with these biologics for 24 weeks. Additionally, we analyzed B cell activation and proliferation based on surface expression of CD95 and intracellular expression of Ki-67, extending our investigation to different B cell subsets and their modulation during cytokine inhibition. Our data revealed an activated phenotype in B cells, particularly in memory B cell subsets, including DN, pre-switch, and post-switch B cells in RA patients (n = 80) when compared with HD (n = 40) at baseline (**Figures 1**, **2**). Compared with HD, these activation and proliferation markers of B cells (CD95 and Ki-67) were significantly higher in RA patients ($p < 0.0001$). Our data did not reveal any differences in B cell activation status in patients treated with TCZ or ADA along with either concomitant DMARD or concomitant steroid. Elevated B cell activation has been reported in autoimmune diseases like SLE and RA, where B cells are considered to play a role in pathogenesis (36, 37). Reportedly, a previous report has demonstrated that the expressions of CD95 and CD86 were up-regulated in the B cells of new-onset RA patients and positively correlated with DAS28 (37). Similar reports in SLE have shown that the CD95 expression on B cells was increased in relation to their activation, and correlated with disease activity (38). Reportedly, SLE patients present higher CD95 expression in CD27+ B cells (39), and CD27-IgD- CD95+B cells were shown to be associated with active disease (40). In our patient cohort, disease activity, as determined by DAS28 and other inflammatory factors like ESR and CRP levels, was elevated. We observed a positive correlation between DAS28 and B cells expressing CD95 and Ki-67 (**Figure 3**). Under cytokine inhibition therapies using anti-IL6R and anti-TNF-α, the higher activity of total B cells and their subsets expressing CD95 and Ki-67 was significantly reduced, along with DAS28 score and other inflammatory factors (ESR, CRP levels; **Table 1**). In B cells, we observed a significant decline in the expression of surface CD95 and intracellular Ki-67 during TCZ (<0.0001) and ADA (<0.001) therapy. During both cytokine inhibition therapies, CD95 and Ki-67 expressions in all B cell memory subsets investigated in the present study were significantly reduced from baseline to week 12 and week 24 (**Figure 2**).

Among the B cell subsets, the post-switch memory B cells demonstrated the highest expressions of CD95 and Ki-67, followed by pre-switch and DN memory B cells. This implies that the overall B cell activation might largely depend on post-switch B cells (**Figure 2**). Previous reports have indicated that anti-TNF treatment influences the expression of the costimulatory molecule, CD86, as well as that of the inhibitory receptor, Fcγ receptor IIb (FcγRIIb) (41), on B cells. Furthermore, our data corroborate with previous reports showing a decreased expression of activation marker CD69, along with an increase in regulatory B cells after ADA (42) and TCZ treatments (14), indicating that cytokine inhibition therapy can normalize peripheral B cells. In this regard, recent functional investigations of RA, SLE, and Sjögren's B cells have identified that particularly memory B cells from patients in an anergic or post-activated status demonstrate reduced B cell receptor responsiveness and cytokine production (43). Most noteworthy and consistent

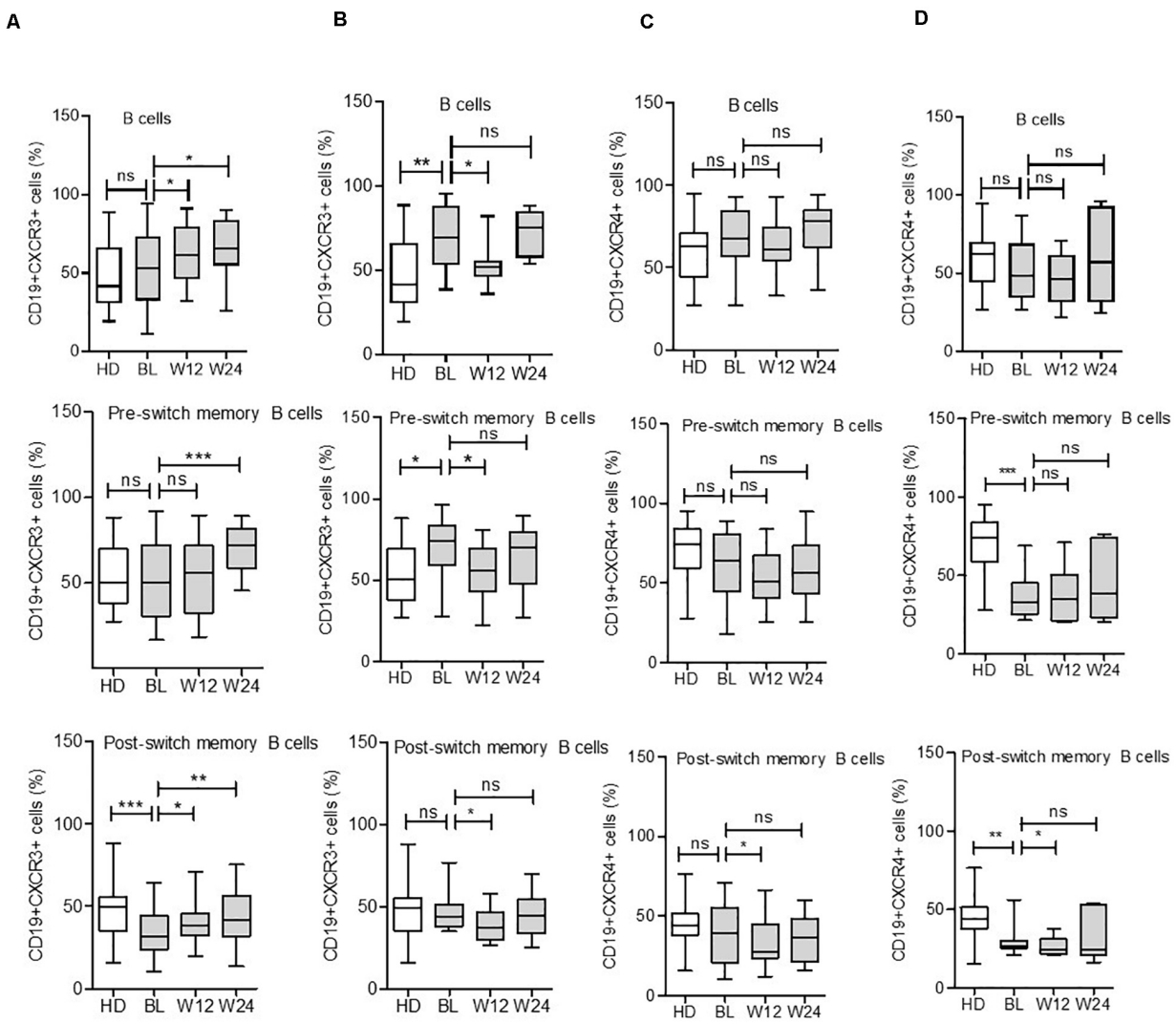

FIGURE 5 | Expression of CXCR3 and CXCR4 in B cell subsets during IL-6R and TNF inhibition therapy. CXCR3 expression on B cell subsets increases in some subsets during IL-6R inhibition **(A)** and decreases during TNF-α inhibition **(B)** therapy. CXCR4 expression on B cell subsets reduces in certain subsets during IL-6R inhibition **(C)** and TNF-α inhibition **(D)** therapy. Values were consistently compared with baseline levels using the Student unpaired *t*-test (***$p < 0.0001$, **$p < 0.001$, and *$p < 0.01$). BL = baseline, W12 = week 12, and W24 = week 24.

with the potential reversal of these functional impairments, TCZ treatment reportedly improves the capacity of cytokine production by B cells in RA (44). These findings suggest that anti-cytokine therapy in patients with RA results in detectable improvements of B cell functions, thus supporting the current findings.

Chemokines regulate cellular migration to various physiological and pathological processes via chemokine receptors, contributing to B cell migration, as well as their proliferation and cytokine production in RA (36). CXCR3 is a chemokine receptor for inflammatory chemokines, including CXCL9, 10, and 11 (45). Higher levels of CXCR3 expressing T cells have been documented in patients with RA and correlate with disease activity (46). CXCR3 knockout mice are reportedly resistant to inflammatory autoimmune disease (47). Compared to HD, we observed a numeric increase in CD19+CXCR3+ expressing B cells in RA patients (**Figure 4**). Interestingly, CD19+CXCR3+ expressing B cells were present in naïve, as well as memory B cell subsets (**Figure 5**). During TCZ treatment, the B cell subset expression of CXCR3 increased, particularly in the post-switch memory compartment; however, during ADA therapy, CXCR3 expressing cells decreased significantly during the first 12 weeks and remained unaltered in later weeks. This data may indicate that cytokine therapies demonstrate differential effects on B cell subsets. As IFN-γ is involved in the induction of CXCR3 expression on B cells (45), we postulate that different cytokine inhibitions induce differential T cell responses, and IL-6R inhibition by TCZ might lead to Th1-biased immunomodulation. Interestingly, IFN-γ producing Th1 cells are elevated in RA patients during TCZ therapy (48),

whereas a decrease in Th1 cells has been documented during ADA treatment (49). Chemokine receptor CXCR4+ B cells demonstrated a higher expression tendency in RA patients when compared with HD (**Figure 4B**). CXCR4 is expressed by B cell subsets throughout the B cell ontogeny and its ligands. CXCL12 is broadly distributed in various tissues (50). CXCR4 is an essential homing receptor and is required for the normal accumulation of plasma cells, as well as for retaining developing B cells in the bone marrow (45, 51). Additionally, CXCR4 and its ligand, CXCL12, are reportedly involved in the pathogenesis of RA (52). Our data showed a marginal reduction in CD19+CXCR4+ B cells during week 12 of treatment; however, this modulation was not statistically significant. In both TCZ and ADA treatment, this reduction was not observed during week 24, indicating the minimal influence of anti-cytokine therapy on CD19+CXCR4+ B cells (**Figure 5**). A detailed analysis of B cell subsets for the expression of CXCR4 demonstrated the significant modulation on CD19+CXCR4+ post-switch memory B cells.

In conclusion, our study revealed a phenotype of activated B cell subsets, particularly in the post-switch B cell compartment, in patients with active RA, and its successful modulation during cytokine inhibition therapies. The higher expressions of surface CD95 and intracellular Ki-67 at baseline reflected the disease activity and are positively correlated with RA disease activity. The subtle change observed in the expressions of chemokine receptors, CXCR3 and CXCR4, indicates that the migratory and homing capacity of B cells might be altered during cytokine inhibition. Our data further contribute to knowledge regarding the therapeutic effects of cytokine inhibitors on peripheral B cells. The B cell activation status may be further explored as predictors of response during such therapies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the University Hospital, Würzburg, Germany and was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZM, H-PT, and KM conceptualized the project. ZM, MS, and KM were involved in data acquisition and analysis. MS was involved in clinical studies and revising the manuscript. TD provided reagents and critically revised the manuscript. KM led the investigation and wrote the manuscript with the help of ZM and H-PT. All authors approved the final version to be published. H-PT and KM had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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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.2020.572475/full#supplementary-material

REFERENCES

1. Vivar N, Van Vollenhoven RF. Advances in the treatment of rheumatoid arthritis. *F1000Prime Rep*. (2014) 6:31. doi: 10.12703/P6-31
2. Pitzalis C. Pathogenesis of rheumatoid arthritis: from systemic autoimmunity to localised joint disease. *Drug Discov Today*. (2014) 19:1152–4. doi: 10.1016/j.drudis.2014.05.009
3. Elemam NM, Hannawi S, Maghazachi AA. Role of chemokines and chemokine receptors in rheumatoid arthritis. *Immunotargets Ther*. (2020) 9:43–56. doi: 10.2147/ITT.S243636
4. Klareskog L, Stolt P, Lundberg K, Kallberg H, Bengtsson C, Grunewald J, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum*. (2006) 54:38–46. doi: 10.1002/art.21575
5. Luque Ramos A, Redeker I, Hoffmann F, Callhoff J, Zink A, Albrecht K. Comorbidities in patients with rheumatoid arthritis and their association with patient-reported outcomes: results of claims data linked to questionnaire survey. *J Rheumatol*. (2019) 46:564–71. doi: 10.3899/jrheum.180668
6. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest*. (2008) 118:3537–45. doi: 10.1172/JCI36389
7. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum*. (2003) 48:2741–9. doi: 10.1002/art.11223
8. Fekete A, Soos L, Szekanecz Z, Szabo Z, Szodoray P, Barath S, et al. Disturbances in B- and T-cell homeostasis in rheumatoid arthritis: suggested relationships with antigen-driven immune responses. *J Autoimmun*. (2007) 29:154–63. doi: 10.1016/j.jaut.2007.07.002

9. Lindenau S, Scholze S, Odendahl M, Dorner T, Radbruch A, Burmester GR, et al. Aberrant activation of B cells in patients with rheumatoid arthritis. *Ann N Y Acad Sci*. (2003) 987:246–8. doi: 10.1111/j.1749-6632.2003.tb06055.x
10. Roll P, Mahmood Z, Muhammad K, Feuchtenberger M, Dorner T, Tony HP. Long-term repopulation of peripheral B-cell subsets after single and repeated rituximab infusions in patients with rheumatoid arthritis. *Clin Exp Rheumatol*. (2015) 33:347–53.
11. Martin F, Chan AC. Pathogenic roles of B cells in human autoimmunity; insights from the clinic. *Immunity*. (2004) 20:517–27. doi: 10.1016/S1074-7613(04)00112-8
12. Barnas JL, Looney RJ, Anolik JH. B cell targeted therapies in autoimmune disease. *Curr Opin Immunol*. (2019) 61:92–9. doi: 10.1016/j.coi.2019.09.004
13. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med*. (2012) 209:1001–10. doi: 10.1084/jem.20111675
14. Snir A, Kessel A, Haj T, Rosner I, Slobodin G, Toubi E. Anti-IL-6 receptor antibody (tocilizumab): a B cell targeting therapy. *Clin Exp Rheumatol*. (2011) 29:697–700. doi: 10.1136/ard.2010.149005.1
15. Narazaki M, Tanaka T, Kishimoto T. The role and therapeutic targeting of IL-6 in rheumatoid arthritis. *Expert Rev Clin Immunol*. (2017) 13:535–51. doi: 10.1080/1744666X.2017.1295850
16. Yoshida Y, Tanaka T. Interleukin 6 and rheumatoid arthritis. *Biomed Res Int*. (2014) 2014:698313. doi: 10.1155/2014/698313
17. Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL. Anti-TNF-alpha therapies: the next generation. *Nat Rev Drug Discov*. (2003) 2:736–46. doi: 10.1038/nrd1175
18. Gran F, Kerstan A, Serfling E, Goebeler M, Muhammad K. Current developments in the immunology of psoriasis. *Yale J Biol Med*. (2020) 93:97–110.
19. deLuca LS, Gommerman JL. Fine-tuning of dendritic cell biology by the TNF superfamily. *Nat Rev Immunol*. (2012) 12:339–51. doi: 10.1038/nri3193
20. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med*. (2001) 344:907–16. doi: 10.1056/NEJM200103223441207
21. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. (2011) 365:2205–19. doi: 10.1056/NEJMra1004965
22. Pala O, Diaz A, Blomberg BB, Frasca D. B lymphocytes in rheumatoid arthritis and the effects of anti-TNF-alpha agents on B lymphocytes: a review of the literature. *Clin Ther*. (2018) 40:1034–45. doi: 10.1016/j.clinthera.2018.04.016
23. Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. High TNF-alpha levels in resting B cells negatively correlate with their response. *Exp Gerontol*. (2014) 54:116–22. doi: 10.1016/j.exger.2014.01.004
24. Voulgari PV, Drosos AA. Adalimumab in the treatment of rheumatoid arthritis. *Expert Opin Biol Ther*. (2014) 14:549–561. doi: 10.1517/14712598.2014.894503
25. Choy EH, Benedetti F. De, Takeuchi T, Hashizume M, John MR, Kishimoto T. Translating IL-6 biology into effective treatments. *Nat Rev Rheumatol*. (2020) 16:335–45. doi: 10.1038/s41584-020-0419-z
26. Carbone G, Wilson A, Diehl SA, Bunn J, Cooper SM, Rincon M. Interleukin-6 receptor blockade selectively reduces IL-21 production by CD4 T cells and IgG4 autoantibodies in rheumatoid arthritis. *Int J Biol Sci*. (2013) 9:279–88. doi: 10.7150/ijbs.5996
27. Emery P, Keystone E, Tony HP, Cantagrel A, van Vollenhoven R, Sanchez A, et al. IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial. *Ann Rheum Dis*. (2008) 67:1516–23. doi: 10.1136/ard.2008.092932
28. Roll P, Muhammad K, Schumann M, Kleinert S, Einsele H, Dorner T, et al. In vivo effects of the anti-interleukin-6 receptor inhibitor tocilizumab on the B cell compartment. *Arthritis Rheum*. (2011) 63:1255–64. doi: 10.1002/art.30242
29. Chen YF, Jobanputra P, Barton P, Jowett S, Bryan S, Clark W, et al. A systematic review of the effectiveness of adalimumab, etanercept and infliximab for the treatment of rheumatoid arthritis in adults and an economic evaluation of their cost-effectiveness. *Health Technol Assess*. (2006) 10:iii–iv, xi–xiii, 1–229. doi: 10.3310/hta10420
30. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis*. (2010) 69:1580–8.
31. Mahmood Z, Muhammad K, Schmalzing M, Roll P, Dorner T, Tony HP. CD27-IgD- memory B cells are modulated by in vivo interleukin-6 receptor (IL-6R) blockade in rheumatoid arthritis. *Arthritis Res Ther*. (2015) 17:61. doi: 10.1186/s13075-015-0580-y
32. Alam J, Jantan I, Bukhari SNA. Rheumatoid arthritis: Recent advances on its etiology, role of cytokines and pharmacotherapy. *Biomed Pharmacother*. (2017) 92:615–33. doi: 10.1016/j.biopha.2017.05.055
33. Smolen JS, Landewe RBM, Bijlsma JWJ, Burmester GR, Dougados M, Kerschbaumer A, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. *Ann Rheum Dis*. (2020) 79:685–99.
34. Dimitrijevic M, Arsenovic-Ranin N, Kosec D, Bufan B, Nacka-Aleksic M, Pilipovic I, et al. Sex differences in Tfh cell help to B cells contribute to sexual dimorphism in severity of rat collagen-induced arthritis. *Sci Rep*. (2020) 10:1214. doi: 10.1038/s41598-020-58127-y
35. Behrens M, Luckey D, Luthra H, David C, Taneja V. B cells influence sex specificity of arthritis via myeloid suppressors and chemokines in humanized mice. *Clin Immunol*. (2017) 178:10–9. doi: 10.1016/j.clim.2015.05.015
36. Henneken M, Dorner T, Burmester GR, Berek C. Differential expression of chemokine receptors on peripheral blood B cells from patients with rheumatoid arthritis and systemic lupus erythematosus. *Arthritis Res Ther*. (2005) 7:R1001–13. doi: 10.1186/ar1776
37. Wang J, Shan Y, Jiang Z, Feng J, Li C, Ma L, et al. High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis. *Clin Exp Immunol*. (2013) 174:212–20. doi: 10.1111/cei.12162
38. Bijl M, Horst G, Limburg PC, Kallenberg CG. Fas expression on peripheral blood lymphocytes in systemic lupus erythematosus (SLE): relation to lymphocyte activation and disease activity. *Lupus*. (2001) 10:866–72. doi: 10.1191/096120301701548517
39. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, et al. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J Immunol*. (2000) 165:5970–9. doi: 10.4049/jimmunol.165.10.5970
40. Jacobi AM, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, et al. Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95. *Arthritis Rheum*. (2008) 58:1762–73. doi: 10.1002/art.23498
41. Catalan D, Aravena O, Sabugo F, Wurmann P, Soto L, Kalergis AM, et al. B cells from rheumatoid arthritis patients show important alterations in the expression of CD86 and FcgammaRIIb, which are modulated by anti-tumor necrosis factor therapy. *Arthritis Res Ther*. (2010) 12:R68. doi: 10.1186/ar2985
42. Banko Z, Pozsgay J, Gati T, Rojkovich B, Ujfalussy I, Sarmay G. Regulatory B cells in rheumatoid arthritis: alterations in patients receiving anti-TNF therapy. *Clin Immunol*. (2017) 184:63–9. doi: 10.1016/j.clim.2017.05.012
43. Weissenberg SY, Szelinski F, Schrezenmeier E, Stefanski AL, Wiedemann A, Rincon-Arevalo H, et al. Identification and characterization of post-activated B cells in systemic autoimmune diseases. *Front Immunol*. (2019) 10:2136. doi: 10.3389/fimmu.2019.02136
44. Fleischer S, Ries S, Shen P, Lheritier A, Cazals F, Burmester GR, et al. Anti-interleukin-6 signalling therapy rebalances the disrupted cytokine production of B cells from patients with active rheumatoid arthritis. *Eur J Immunol*. (2018) 48:194–203. doi: 10.1002/eji.201747191
45. Muehlinghaus G, Cigliano L, Huehn S, Peddinghaus A, Leyendeckers H, Hauser AE, et al. Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood*. (2005) 105:3965–71. doi: 10.1182/blood-2004-08-2992
46. Motoki Y, Tani K, Shimizu T, Tamiya H, Hase K, Ohmoto Y, et al. The expression of chemokine receptor CXCR3: relevance to disease activity of rheumatoid arthritis. *Mod Rheumatol*. (2003) 13:114–20. doi: 10.3109/s10165-002-0209-2
47. Karin N, Razon H. Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. *Cytokine*. (2018) 109:24–8. doi: 10.1016/j.cyto.2018.02.012

48. Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, Lopez MN, et al. Effect of interleukin-6 receptor blockade on the balance between regulatory T cells and T helper type 17 cells in rheumatoid arthritis patients. *Clin Exp Immunol*. (2013) 171:237–42. doi: 10.1111/cei.12017
49. Aravena O, Pesce B, Soto L, Orrego N, Sabugo F, Wurmann P, et al. Anti-TNF therapy in patients with rheumatoid arthritis decreases Th1 and Th17 cell populations and expands IFN-gamma-producing NK cell and regulatory T cell subsets. *Immunobiology*. (2011) 216:1256–63. doi: 10.1016/j.imbio.2011.07.006
50. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J Exp Med*. (2004) 200:1145–56. doi: 10.1084/jem.20041185
51. Becker M, Hobeika E, Jumaa H, Reth M, Maity PC. CXCR4 signaling and function require the expression of the IgD-class B-cell antigen receptor. *Proc Natl Acad Sci USA*. (2017) 114:5231–6. doi: 10.1073/pnas.1621512114
52. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature*. (2003) 423:356–61. doi: 10.1038/nature01661

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REVIEW
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Immunoglobulin M in Health and Diseases: How Far Have We Come and What Next?

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B lymphocytes are important in secreting antibodies that protect against invading pathogens such as viruses, bacteria, parasites, and also in mediating pathogenesis of allergic diseases and autoimmunity. B lymphocytes develop in the bone marrow and contain heavy and light chains, which upon ligation form an immunoglobulin M (IgM) B cell receptor (BCR) expressed on the surface of naïve immature B cells. Naïve B cells expressing either IgM or IgD isotypes are thought to play interchangeable functions in antibody responses to T cell-dependent and T cell-independent antigens. IgM short-lived plasma cells (SLPCs) and antigen-specific IgM memory B cells (MBCs-M) are critical in the first few days of infection, as well as long-term memory induced by vaccination, respectively. At mucosal surfaces, IgM is thought to play a critical part in promoting mucosal tolerance and shaping microbiota together with IgA. In this review, we explore how IgM structure and BCR signaling shapes B cell development, self and non-self-antigen-specific antibody responses, responses to infectious (such as viruses, parasites, and fungal) and non-communicable diseases (such as autoimmunity and allergic asthma). We also explore how metabolism could influence other B cell functions such as mucosal tolerance and class switching. Finally, we discuss some of the outstanding critical research questions in both experimental and clinical settings targeting IgM.

Keywords: immunoglobulin M (IgM), B cell development, short-lived plasma cell (SLPC), long-lived plasma cell (LLPC), memory B cell (MBC)

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INTRODUCTION

IgM is the first antibody isotype expressed during B cell development and the first humoral antibody responder, conserved across all species from Zebrafish to humans (1). In cartilaginous and bony fish, IgM has been found to have crucial functions in host defense and tolerance (2). IgM can be divided into natural and antigen-induced IgM and can either be membrane bound IgM-type BCR or secreted IgM (3, 4). Natural IgM plays multiple roles in homeostasis including scavenging, B cell

tonic signals for B cell survival, lymphoid tissue architecture, and prevention of autoimmune diseases (5, 6). IgM is involved in clearance of debris, particles (below 2 μM) and apoptotic cells through antibody dependent opsonization and phagocytosis by macrophages (7, 8). At mucosal sites both natural and antigen-induced IgM play a role in shaping healthy microbiota and their repertoire, although limited, is also shaped by microbiota (9, 10). Secreted IgM antigen-complexes can connect signals *via* unique and shared receptors, suggest a more pleotropic role in homeostasis and disease states (11, 12).

Since the discovery of individuals with selective IgM deficiency, a lot has been learnt about IgM in various human diseases including autoimmune and infectious diseases (13, 14). Genetically conditioned mice which lack secreted or membrane bound IgM have underscored the importance of IgM in many infectious diseases. In this review, we highlight what is currently known about the role of IgM in B1 and B2 cell development, memory, and plasma cell generation, in and outside GCs. Lastly, we discuss experimental models using IgM-deficient mice and corroborating phenotypes observed in humans with selective IgM deficiency.

B CELL DEVELOPMENT

Naturally Occurring Immunoglobulin M B Cells (B1)

B1 cells develop in the yolk sac on embryonic day 9, before birth from a functional hematopoietic stem cell subset termed the common lymphoid progenitor, in the fetal liver and seed the peritoneal and pleural cavities (15–21). B1 cells are thought to be the main source of naturally occurring IgM, although there is controversy on the main contributing organ, with some studies suggesting bone marrow (BM) and spleen B1 cells as important sources (22). B1 cells are thought to lack specificity and affinity maturation similar to innate immune receptors and are referred to as innate-like B cells or unconventional (4, 16). The concept of non-specificity is somewhat nullified by the fact that B1 cells are polyreactive—they recognize polysaccharides found on the cell wall surfaces of a wide array of pathogens, but with exquisite specificity (23, 24). This specificity allows them to confer protection against pathogens bearing similar epitopes (discussed later). Furthermore, B1 cells are self-reactive and develop normally in the absence of foreign antigen stimulation, suggesting that their development is self-regulated *via* a mechanism of binding to glycosylated and oxidized mammalian molecules to prevent self-recognition (15, 20, 25). B cell receptor is intricately regulated by CD5 (Ly1) which enables self-antigen recognition and some level of specificity (**Figure 1A**) (20, 26).

The majority of B1 cells are found in the peritoneal cavity where they are self-renewing and undergo maintenance with the help from resident macrophages that secrete CXCL13 (27). Other sites such as spleen, lymph node, bone marrow, pericardium, and mucosal associated lymphoid tissue account for as little as 1% of B1 total pool (11, 22, 28, 29). The phenotype of B1 cells varies depending on the compartment, with splenic B1 cells and peritoneal B1 cells displaying different antibody repertoire, gene expression, and secretion of IgM (16). In the peritoneal cavity, B1 cells can be identified by surface expression of $CD19^{hi}$, $B220^{low}$, $CD43^{+}$ $CD5^{+}/CD5^{low/-}$, $CD23^{low}$, $CD11b^{+}$, whereas in other tissues, where they migrate after injury, they lose CD11b expression as they become plasma cells, making it difficult to differentiate them with B2 cells in these tissues (16, 26). B1 cells are divided into B1a ($CD5^{+}$) and B1b ($CD5^{-}$), with B1a cells accounting for the majority of the B1 cell population (16, 20, 25, 30, 31). While B1b cells can potentially develop from bone marrow progenitors, B1a cells cannot (30, 32). Both B1a and B1b cells display similar surface markers with the exception of CD5, which regulates B1a cell autoreactivity (16, 26).

Bone Marrow Derived Immunoglobulin M B Cells (B2)

Conventional B cells (B2) are derived from bone marrow after birth from a common lymphoid progenitor (CLP) and their commitment to B cell lineage is dependent on the BM microenvironment (25) (**Figure 1B**). B cell lymphopoiesis is a rather complex process. Here, we give a brief summary mainly to illustrate how naïve B cells exiting the BM expressing surface IgM reach peripheral tissues. For more detailed reviews on this topic, we refer the reader to a number of review articles (33–35). The subsequent stages are important in a B cell's development and they introduce diversity into the antibody's repertoire (36). The first stage is a pre-pro B cell, where initial diversification of the D and J segments occurs, followed by the pro-B cell where recombination of the V region to the previously rearranged D-J is completed (33–36). Interleukin 7 (IL-7) from stroma and IL-7Rα signaling on developing B cells play both positive and negative regulatory roles in B cell development, allowing proliferation and pro-survival signals, as well as switching off recombination for next stage of development (34, 37). Following a successful V-D-J rearrangement in the pro-B cell, expression of the Igμ heavy chain (μHC) in the pre-B cell stage occurs. The V and J segments of the two germline-encoded surrogate light chain (VpreB and lamda5), combine with an existing Igμ heavy chain (33). This is then followed by association with signaling subunits Igα and Igβ and assembly, resulting in surface expression of the pre-BCR (34–36). The pre-B cells are large and motile and act in positive selection to select against autoreactivity, making the pre-B cell stage a tolerance checkpoint (33, 38, 39). Recombination activating genes 1/2 (RAG1/RAG2) are key in the progenitor B cell development and allow genetic recombination rearrangement (40). The final transition of these large proliferating pre-B cells before they exit the BM occurs as they move away from the IL-7 rich stromal region, downregulate IL-7Rα and induce the expression of the IRF4 transcription factor (37, 41). IRF4 induces transcription of CXCR4, which in turn inhibits proliferation and cell cycle exit, as well as inducing reduction in size of the pre-B cell. RAG1/RAG2 allow for a final recombination of the V and J regions of the light chain (Igκ and IgL) in the CXCL12-CXCR4 rich environment and development of the immature B cell (25)

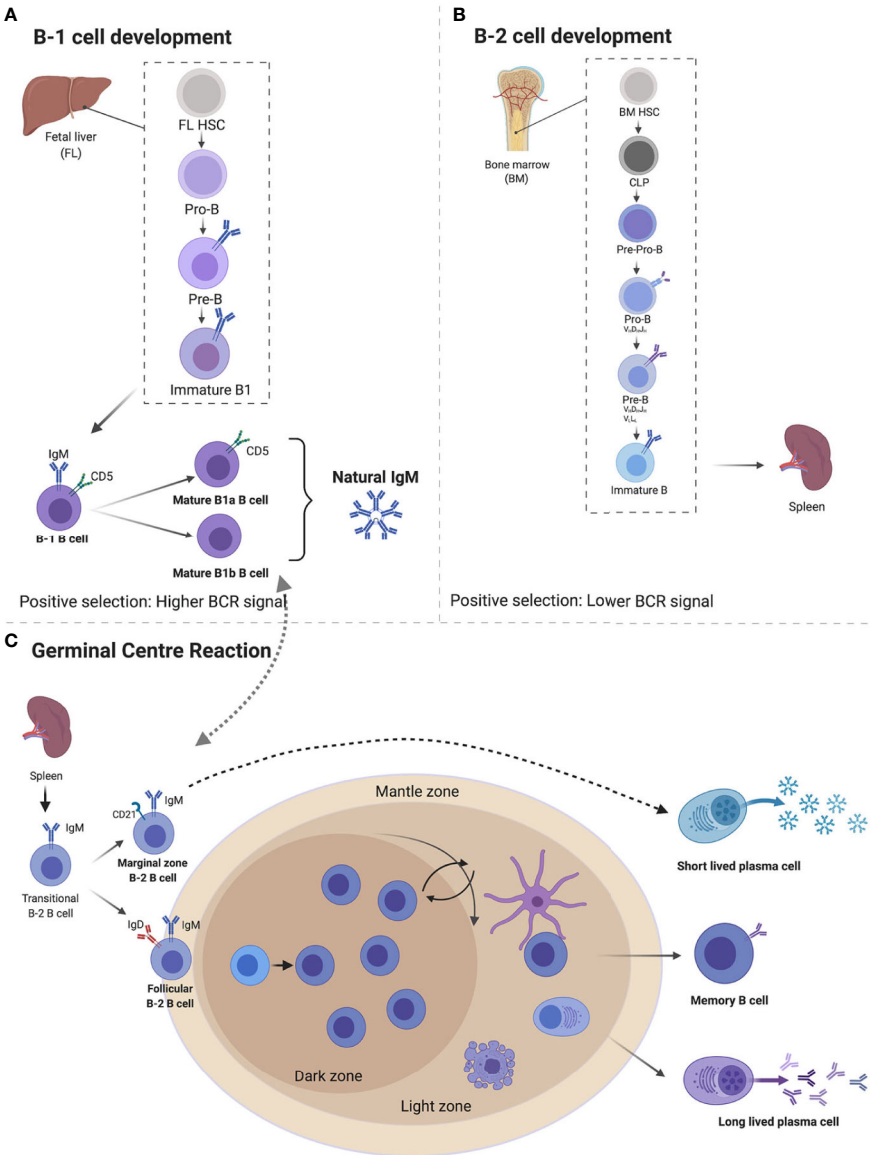

FIGURE 1 | Immunoglobulin M (IgM) developmental pathways through B1 and B2 B cells from fetal liver (FL) and bone marrow (BM). B1 cells develop FL where they go through pro-B cell, pre-B cell, immature B cell, and naïve B cells expressing IgM and CD5 which differentiates B1a and B1b cells, both capable of secreting natural IgM **(A)**. B2 cells develop from BM's common lymphoid progenitor to become immature B cells that migrate to splenic B cells secreting IgM. Expression of IgD differentiates marginal zones *vs.* follicular B cells **(B)**. Follicular B cells upon antigen stimulation can either undergo germinal center maturation creating long-lived plasma cells, memory B cells, class switch, or remain unswitched short-lived plasma cells **(C)**. Created with BioRender.com.

(**Figure 1B**). The immature B cells leave the BM *via* vascular sinuses and migrate to the peripheral tissues such as the spleen and lymph nodes where they complete their final maturation (38).

Peripheral B Cell Maturation and Production of Immunoglobulin M by B2 Cells

The regulation of B cell development is mediated by the BCR when transitioning from an immature to a mature B cell (25). An immature transitional B cell undergoes several splicing events and primary variable diversity joining of Cμ and Cδ transcripts (42, 43). This leads to a naïve B cell co-expressing both IgM and IgD BCRs isotypes on the surface, with identical specificities (43, 44). These naïve B cells still display a certain level of self-reactivity and are further pruned through clonal deletion and anergy, where they can become unresponsive to self-antigen stimulation, thus preventing autoimmunity (25, 42, 44–46). Transitional B cells localize in secondary lymphoid tissues such

as the spleen or lymph nodes, where they spatially sub-localize in follicular regions for easy access to both sampling of antigens and a local area rich with B cell survival factors, such as BAFF (25, 46). The naïve B cells are attracted to follicular areas by CXCL13 chemokines and once they encounter antigens, they upregulate CCR7, which enables them to sense CCL21- and CCL19-rich T cell zone areas (46). At this stage, B cells seek T cell help for a cognate antigen, which further stimulates their survival, proliferation, and antibody secretion function (47). In order for antigen primed naïve B cells to have access to highly competitive T cell help, they need to undergo several rounds of high affinity maturation to create clones that are likely to survive longer and possibly create long term memory (**Figure 1C**). These processes take place in the germinal centers (GCs), which are secondary B cell follicle areas (47, 48). Naïve B cells that do not to take part in the GC reaction are pushed to the B cell mantle zone, where they divide and form short-lived plasmablasts, which eventually produce low affinity short-lived IgM plasma cells. The GC [identified by GL7 and Fas (CD95) expression] is a highly proliferative area, divided into the light zone (LZ) and dark zone (DZ) (48). The LZ contains follicular dendritic cells (FDCs), where selection of BCRs takes place (48). The B cells receive the antigen from FDCs, present it to T follicular helper (Tfh) cells; if the mutation confers an advantage, the specific cell will be selected (48, 49). The DZ is the area in which where somatic hypermutation (SHM) takes place and it appears dark, due to the densely packed B cells that proliferate (**Figure 1C**) (47, 48). In the DZ, *Aicda*, a gene that encodes for activation-induced deaminase (AID) is highly expressed. AID deaminates cytidine residues in the VDJ and switch regions of the Ig gene, leading to SHM and class switch (47, 50). During SHM, AID catalyzes the deamination of C to U, to activate error prone repair pathways to induce mutations (51, 52).

Class switching, which occurs in the GC and occasionally in extrafollicular sites (47, 53), involves the replacement of the H-chain C-region for another Ig gene, for example mμ (IgM) for gamma (IgG) (36). The constant region (Fc) of the BCR changes, while the variable side (Fab) remains constant, therefore the antigen specificity prevails. However, various signaling cascades and immune responses occur, based on the class of Ig that is present. Within the GC population, IgG/IgM cells ratio remains constant, indicating a dynamic steady state between class switched and non-class switched cells (53). The process of antibody class switching is evolutionary conserved across species and is found as early in evolution as cartilaginous sharks and *Xenopus* (54). In the South African clawed frog (*Xenopus laevis*), IgM shows limited antibody repertoire and reduced affinity despite reasonable mutation rates compared to mammals (54). The limiting factors for IgM affinity in clawed frogs and sharks appear to be a lack of germinal center (GC) B cell compartment, as well as reduced AID-dependent somatic hypermutations that are found in mammals (54, 55). This limited mutation rate is at least partially evolutionary conserved, as it is observed in certain long-lived memory IgM B cells or low affinity memory B cells generated outside GCs in humans (56, 57).

STRUCTURE OF IMMUNOGLOBULIN M AND ANTIGEN RECOGNITION

IgM exists in two forms—membrane bound (mIgM) and secreted (sIgM), with sIgM being further divided into natural and antigen induced IgM (**Figure 2**) (5, 6). IgM can exist in various structural forms including a monomer, a hexamer, and a pentamer, the latter weighing over 1,000 kDa (6, 58). Pentameric assembly of sIgM is the most naturally occurring form, with monomers held together by a 15-kDa protein J-chain that bridges disulfide bonds *via* a C-terminal extension of the heavy-chain (**Figure 2B**) (59–62). IgM typically displays low binding affinity to antigens, however, the multivalent antigen-binding sites in the pentameric structure of sIgM and its multivalent antigen-binding sites lead to high avidity for antigens, ensuring efficient elimination of pathogens (63, 64). Similar to other antibody structures, IgM BCR is composed of two homodimeric heavy chains, each bearing a light chain linked *via* disulfide bonds (65, 66). The μ region of the heavy chain folds into four domains, with the constant μ domain 4 (Cμ4) allowing anchoring of the membrane bound IgM to the surface of the B cell and activation of complement (**Figure 2A**) (67, 68). The membrane bound IgM BCR is essential for B cell development and activation, *via* the phosphoinositide 3-kinase pathway (**Figure 2A**) (69–71). The role of hexameric IgM structure is currently unclear, but it is thought to exist due to defects in the μ chain or J-chain regions in pentameric IgM (72). Secretion of IgM is regulated by the secretary component (SC) and J-chain (**Figure 2A**), which regulate surface availability of IgM and premature release through preventing protease cleavage, particularly in mucosal sites where there is richness in microbes that often use these mechanisms to evade host recognition (61). Apart from regulation by SC and J-chain, sIgM is also post-translationally modified through N-glycosylation and sialylation (60, 73). Most of the N-glycosylation sites are in the μ chain and with one site in the J-chain and mutations in these sites lead to accumulation of IgM on the cell surface and reduced secretion (73, 74).

Upon binding to surface-exposed antigens *via* antibody binding region (Fab), pentameric IgM complexes undergo conformational changes (68) followed by interaction of the antibody-antigen complex with B cells receptors *via* binding of the constant (Fc) domain. IgM can bind to several cell surface receptors including complement receptor CR2 and CR3, polymeric Ig receptor (pIgR), Fcα/μR and FcμR on B-cells, epithelium cells, and antigen presenting cells (75–77). FcμR specifically binds sIgM in mice and exclusively so in human (77). Mice deficient in FcμR expression exhibit spontaneous GC formation, long-lived plasma cell development and memory B cell formation (76, 78). The polymeric Ig receptor is expressed at the basal membrane of mucosal epithelium and exocrine glands and binds to sIgM and sIgA to mediate transcytosis of these antibodies from lamina propria or ileum to apical mucosal sites where they bind to microbiota (**Figure 3**) (79, 80). Fcα/μ receptor (Fcα/μR) is expressed in non-hematopoietic cells and by marginal zone B-2 cells (81, 82). Binding of IgM-antigen

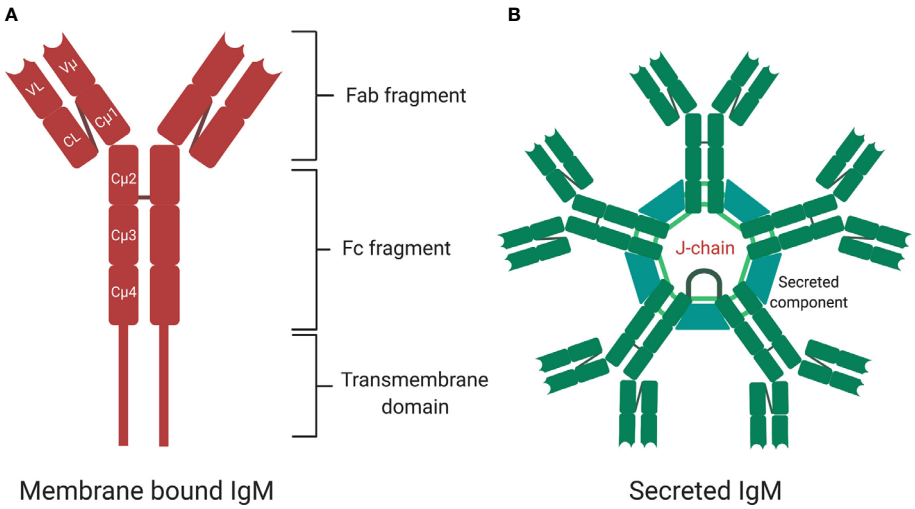

FIGURE 2 | The structure of membrane bound and secreted immunoglobulin M (sIgM). **(A)** A monomer structure of immunoglobulin M (IgM) contains Fab fragments, Fc fragment, and transmembrane signaling tail that attaches to Fc receptors on the surface of B cells. A monomer is made up of two heavy chains and two light chains. **(B)** A pentamer structure is the most naturally occurring form with five monomers held together by a J-chain. Secretary component regulates surface availability and secretion of the pentamer. Created with BioRender.com.

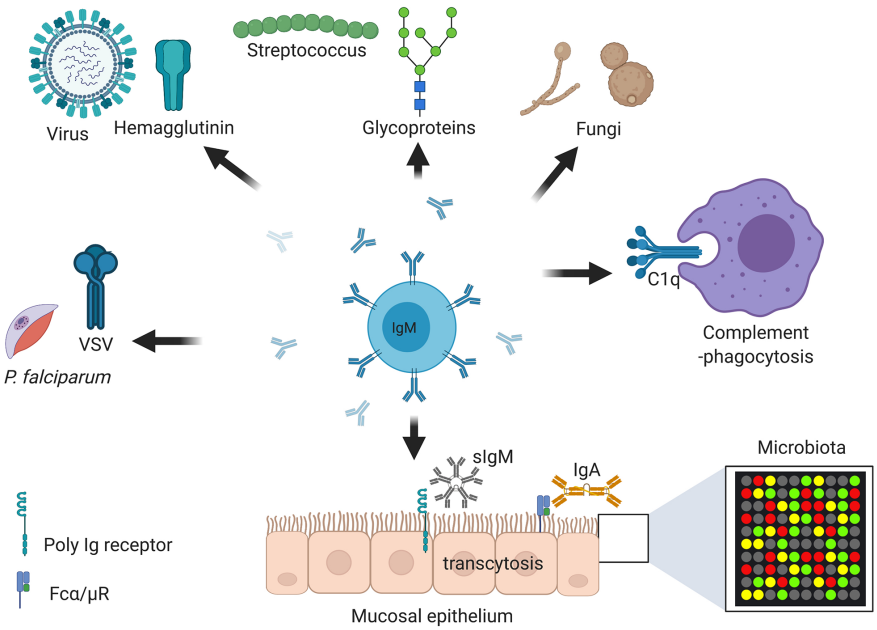

FIGURE 3 | Immunoglobulin M (IgM) is central at steady stage and against infections and non-communicable diseases. Secretory IgM is important at mucosal surfaces in maintenance of healthy microbiota together with secreted IgA. Secretory IgM together with IgM B cell receptor are important in initiation protective immunity against various respiratory pathogens including species of fungi, viruses, and bacteria. Secreted IgM is essential in parasitic infections including those causing malaria and sleeping sickness. Secreted IgM play an important part in cancers diagnosis and auto-immunity diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Secreted IgM has high affinity for C1q, a complement component that allows degradation of antibody coated pathogens and apoptotic debris. Created with BioRender.com.

complexes to the Fcα/μR has been shown to mediate endocytosis and pro-inflammatory cytokine production (81, 82).

IMMUNOGLOBULIN M ANTIBODY RESPONSES TO T CELL-DEPENDENT AND -INDEPENDENT ANTIGENS

Class switching, which occurs in the germinal centers (GCs) and occasionally in extrafollicular sites (36, 47, 53), involves intrachromosomal rearrangement of the Ig heavy chain C-region from Cμ (IgM)/Cδ (IgD) with Cγ1 (IgG) without altering specificity to immunizing antigen (36, 52). This class switching is thought to occur sequentially in GCs (83–87). However, direct class switching from Cμ to Cε or Cα has been observed, particularly in extrafollicular GCs where it is partly thought to be driven by lack of B cell maturity or low activation threshold (83, 88, 89). Class switch recombination is initiated by AID, which targets intronic switch (S) regions, causing DNA breaks and recombination of the heavy chain VDJ segments with other constant regions (50, 51, 84, 87). It is widely thought that memory B cells are generated from antigen T-dependent interactions that take place in the GC and that the majority of first wave plasma cells are of IgM isotype, short-lived, display high avidity and are T-independent (53). Over the last decade, increasing evidence suggests that memory B cells of IgM isotype exist and that these cells can secrete long-lived plasma cells (LLPCs) when stimulated by a cognate antigen (57). The nature of the generation of IgM memory B cells generation is rather complex, as it seems to depend on the tissue of origin (local events), GCs or extrafollicular GCs pathway and SHM rate of integrative genomics viewer (IgV) region of B cell receptor (57, 88, 90–93).

Antigen-Specific Immunoglobulin M Short-Lived Plasma Cells

Short-lived plasma cells (SLPCs) of IgM producing antibodies are typically found in the spleen on the periphery of B cell follicles, displaying little to no SHM (**Box 1A**) (92). Long-lived plasma cells on the other hand, show some degree of high affinity, suggestive of having gone through GCs, and, can be found in the BM (**Box 1B**) (91). Short-lived plasma cells' differentiation is governed by the B-cell lymphoma 6 (BCL6) and PR domain containing 1 (PRDM1)/BLIMP-1 transcription factor (94, 95). BCL6 favors GC entry, whereas BLIMP-1 represses BCL6 and favors antibody secreting cells (ASCs). Interactions showing poor strength between BCR and antigen favor higher avidity, tend to be generated in extrafollicles and do not enter GCs resulting in SLPCs (94, 96). These SLPC release the early wave of antibodies post-antigen exposure and provide the initial protective response prior to emergence of high affinity antibodies (94, 96). Additional evidence suggests a key role for the glycolysis pathway in this T-independent SLPC production (97). This process involves mechanistic target of mTOR activating transmembrane activator and CAML interactor (TACI) *via* MyD88, to induce MZ B cell proliferation and genetic recombination, allowing non-GC class switching (97). LLPCs of IgM isotype were only described recently and differ from IgG LLPCs, as they develop independently of GCs (57). This population persists in the spleen, unlike IgG BM-residing LLPCs and undergo SHM (some outside of the GC), in an AID-dependent and BLC6-independent manner (57, 98, 99). The mutations that occur are not typically in the complementarity determining region 3 (CDR) and are therefore not thought to be selected for by antigen affinity (57). The IgM LLPCs are capable of conferring protection against viral and bacterial infections *in vitro* and *in vivo*, independently of IgG LLPCs, memory B cells, and T cell help (32, 57).

Antigen-Specific Immunoglobulin M Memory B Cells

Antigen specific IgM memory B cells (MBCs-M) form a subset of memory B cells that secrete IgM in the spleen, surprisingly also in germ-free mice, albeit with reduced diversity (**Box 1C**) (56, 100). MBCs were initially described as being IgG or IgA isotypes and expressing high levels of CD73, CD80, and PD-L2. However, it is now accepted that an MBC-M population exists from an early GC reaction and lacks classical MBCs surface molecules (100–102). MBCs-M show poor affinity compared to MBCs-G and contain less IgV mutations, however, their half-life is significantly longer (99). The mechanisms by which MBC-Ms survive longer and are more persistent remain largely unclear. BCR avidity and usage (CDR3 *vs.* non-CDR3) and mouse genotype rather than antigen are thought to be key in the persistence (95). Although, MBC-M are in many ways similar to naïve B cells, they show different dynamics in GC entry and ASCs production (103). Compared to MBCs-M, naïve B cells express considerably higher levels of Krüppel-like factor (KLF) 4, KLF9, and promyelocytic leukemia zinc finger (PLZF), transcription factors associated with quiescence (104). It is likely that these factors repress genes associated with survival and cell cycle, allowing significantly faster turnaround in ASC production and if needed, generation of class switched plasma

BOX 1 | Key differences in effector B cell subsets.

a. Short-lived plasma cells (SLPCs)—SLPCs of IgM producing antibodies are typically found in the spleen on the periphery of B cell follicles, displaying little to no SHM. SLPCs differentiation is governed by the BCL6 and BLIMP-1 transcription factor. SLPC release the early wave of antibodies post-antigen exposure and provide initial protective response prior to emergence of high affinity antibodies.

b. Long lived-plasma cells (LLPCs)—LLPCs continuously secrete antibodies at a constant titre. LLPCs also appear to be more stringently selected and appear in late GCs. LLPCs reside in BM, spleen, and gut-associated lymphoid tissues (GALTs).

c. Memory B cells IgM (MBCs-M)—MBCs secrete antibodies in response to cognate antigen challenge. MBCs maintain a higher diversity and appear much earlier in GCs. MBCs can be tissue resident or are found recirculating secondary lymphoid organs. MBCs-M display a lower mutation load compared to their CSR counterparts. MBCs-M display large cross-reactivity, particularly against conserved N-glycans of bacteria and retroviruses.

cells (94). An additional important aspect that has emerged as key in class switching and plasma cell generation is metabolism (105). A recent study showed that naïve follicular B cells entering GCs prefer fatty acid oxidation over glycolysis as an energy source (106). It is likely that differential metabolite needs may have further upstream implications, particularly in MBCs-M function, be it ASC production or re-entry into GCs for further SHM.

MBCs-M acquire high affinity BCRs through SHM upon re-entry of the cells into GCs in an activation-induced deaminase (AID)-dependent process (90, 100). Earlier studies using a less complex (4-hydroxy-3-nitrophenyl)acetyl (NP) antigen suggested that in the secondary responses, high affinity MBC-Ms matured and were able to become ASCs after booster immunization (107). More recent findings suggest that highly mutated and high affinity MBCs-M do not differentiate into ASCs, a process that is left for low affinity MBC-M in the primary immune response (92). It is likely that high affinity MBC-Ms secreting high affinity IgM have an important role in inflammatory and autoimmune disease such as rheumatoid arthritis (discussed in section 5.1) (108). It is speculated that high affinity MBCs-M class switch to other isotypes, as seen in tissue resident Fc Receptor Like 4 ($FcRL4^+$) fractions in secondary lymphoid organs (SLO) and IgA plasma cells in the gut associated lymphoid tissue (GALT) (88, 92, 100). However, a recent study contradicted this notion and suggested that MBCs are unlikely to re-enter GCs in secondary responses for further diversification (102). It may be reasonable to speculate that the low affinity MBCs-M re-enter GCs for further mutation acquisition, to become high affinity MBCs-M with those that fail to do so becoming ASCs, whereas high affinity MBCs-M either contribute to the memory pool or class switch outside GCs as suggested recently (109). Whether high antigen valency, a feature of pentameric IgM, is a major contributing factor in decision making between high affinity MBCs-M and low affinity MBC-Ms is a fascinating area of research that needs further exploration (110).

In addition to MBCs-M, fate mapping studies using AID have also identified other subsets of MBCs-M in the spleen that spontaneously develop under germ-free conditions and are not derived from BM or gut (56). These MBCs-M display a lower mutation load compared to their class switch recombination (CSR) counterparts, suggesting residual antigen activation in the gut, from potential endogenous or food antigen (56). Additionally, they display large cross-reactivity, particularly against conserved N-glycans of bacteria and retroviruses (56). These MBCs-M display unmutated V_H genes with antibacterial activity, suggesting a pre-programmed antibody immune repertoire (56).

In humans, unswitched IgM memory B cells exist and are more abundant in local tissues such as GALT, lung, and SLOs compared to mice (88). MBCs-M have also been found in blood circulation (identified as $IgM^+IgD^+CD27^+$) and show clonal relatedness to gut specific MBCs-M, IgM only PCs, and IgA only PCs (91, 111). Human gut IgM responses may involve IgM diversification from pre-existing $IgM^+IgD^-CD27^+$ memory specificities, rather than *de novo* recruitment of naive $IgM^+IgD^+CD27^-$ B cells, ensuring considerably faster CSR and providing protection to blood borne infections, possibly through cross-reactivity (91, 111). A recent study, which reported severe infections of *Klebsiella* in immunocompromised patients showed that these patients harbored *Klebsiella* LPS-O3 antigen specific MBCs in peripheral blood which showed clonal relatedness with intestinal plasmablasts (112). These MBCs were mostly MBCs-M, however, MBCs-G and MBCs-A were also found in circulation and closely related to IgA found in the lamina propria. Both MBCs-G and MBCs-A showed higher mutation rates (between 20 and 25 bp/IgHV gene) in their heavy chain variable regions, whereas MBCs-M showed less mutations (around 10bp/IgHV gene) in their VH (112). These antibodies were glycan-specific and bound to O3 antigen of the mannose residues present at the surface of other microorganisms, such as *Saccharomyces cerevisiae*, HIV and several other $Gram^+$ and $Gram^-$ human commensals (112). This is consistent with other studies showing human MBCs-M secreted IgM targeting mucus-embedded SIgA coated commensals in the ileum, thus assisting in providing protection from diverse bacteria (88). These MBCs-M are not limited to bacterial species and have been found in the blood of healthy adults mildly infected with human BK polyomaviruses (113). In such settings, MBCs-M were shown to have high viral neutralizing abilities against BK virus and were also pan-reactive against another related JC virus, which causes progressive multifocal leukoencephalopathy in immunocompromised individuals (113). Interestingly, these MBCs-M were functionally distinct from MBCs-G, lost their neutralizing functionality when Cμ was replaced by Cγ and were resistant to class switching to IgG producing cells (113).

MBCs are different to LLPCs in several ways—LLPCs continuously secrete antibodies at a constant titer, while MBCs only do so in response to cognate antigen challenge (101). Additionally, LLPCs reside in BM, spleen, and gut-associated lymphoid tissues (GALTs), whereas MBCs can be tissue resident or are found recirculating secondary lymphoid organs (SLOs) (94). LLPCs also appear to be more stringently selected and emerge in late GCs, whereas MBCs maintain a higher diversity and appear much earlier in GCs (103, 114). The higher diversity of MBCs provides an evolutionary advantage to the host where there is increased antibody breadth protection, a phenomenon that is critical in most antibody-based vaccine designs.

IMMUNOGLOBULIN M IN DISEASES

Immunoglobulin M in Non-Communicable Diseases

An additional aspect where natural and antigen-induced IgM are thought to play non-redundant roles are autoimmune diseases and cancer. In autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), IgM and IgG titers are increased and associated with disease pathogenesis. In SLE, IgG autoantibodies directed against double stranded DNA (dsDNA) are thought to be pathogenic, while IgMs anti-dsDNA are thought to be protective (115). SLE patients are

typically treated with B-cell depletion therapy, rituximab, with adverse outcomes of hypogammaglobulinemia linked to increased infections in these patients (116). In two studies using SLE prone mouse strains (MRL-lpr/lpr) and NZB x NZW that spontaneously develop SLE (characterized by severe immune complex-mediated glomerulonephritis and death by 12 months of age from renal failure), secreted IgM (sIgM) was shown to be essential in preventing disease (**Table 1**) (137, 138). When lpr mice were crossed with sIgM-deficient mice, they developed a severe form of the disease with increased glomerular immunocomplex deposition and IgG ds-DNA autoantibodies, which was rescued by treatment with IgM autoantibodies (138). In the second study, treatment of NZB x NZW mice IgM anti-dsDNA improved disease symptoms including reduction in renal pathology and organ damage (137).

In cancer, natural IgMs are associated with recognition and removal of precancerous cells, owing to their ability to recognize self-antigens of carbohydrate patterns and quickly activate the complement (7). The presence of natural IgM against specific sugar moieties not found in non-cancerous cells is also used as a diagnostic and a prognosis marker, particularly for breast cancers (**Figure 3**) (139). Some of these recognized sugar moieties include MUCIN 1 (140), SAM6/GPR78 (141), and PAM-1 (142), and have been proving to be useful as prophylactic and therapeutic targets when derived directly from a patient's tumor cells (141, 142).

Very little is known about the role of natural and induced IgM in asthma, despite overrepresentation of asthma in patients with selective IgM syndrome (143, 144). Previous studies have suggested that neonatal vaccination with bacterial species, such as group A streptococcus containing GlcNAc or β-1,3-glucans can protect adult mice against *Aspergillus fumigatus* induced allergic asthma (**Table 1**) (10, 23, 124). Passive immunity with anti-GlcNAc natural IgM antibodies in adult mice protects against developing asthma, suggesting that these conserved germline-encoded IgM antibodies can have broad protective effects against other common allergens containing GlcNAc moieties, such as dermatophytes (124). B1 cells secreting IgM are also known to be stimulated by IL-5 and proliferate in an IL-33 receptor dependent manner (145). In this setting, IgM producing B1 cells promote oxazolone induced contact dermatitis in mice (145). Currently, it is unclear whether natural or secreted IgM plays different roles compared to membrane bound IgM, which is more likely to undergo class switching to IgE. More studies are needed to decipher the function of IgM in asthma beyond class switching.

Immunoglobulin M in Shaping Mucosa Tolerance and Against Bacterial Infections

Microbiota colonize the mucosal sites soon after birth in humans and play key roles in homeostasis (146). The dominant antibodies found at mucosal sites are secretory IgAs, which binds and shapes microbiota (147–149). The majority of IgA plasma cells are generated from memory IgA B cells that reside in the lamina propria (LP) in the gut (150). In addition to IgA, emerging evidence places secreted IgM as a key player in maintaining local homeostasis at mucosal sites, such as the gut and lung, and assists in shaping local microbiota (9, 88). Here, we briefly discuss how local secreted IgM produced by memory IgM B cells shapes microbiota (as discussed under antigen-specific

TABLE 1 | Role of immunoglobulin M (IgM) in infectious and non-infectious diseases.

Organism (disease)	Species	Function	Reference
Plasmodium (malaria)	*P. falciparum*	Anti-α-gal IgM antibodies protective in adolescence	(117)
	P. chabaudi	Anti-α-gal IgM antibodies protective when transferred to mice	(117)
	P. berghei	MBCs-M secrete high affinity IgM in GCs	(118)
Trypanosomes (trypanosomiasis)	*T. brucei brucei*	nIgM not protective, sIgM-deficient mice not susceptible	(119)
	T. congolense	nIgM not protective, sIgM-deficient mice not susceptible	(119)
	T. evansi	nIgM important for primary and secondary responses	(120)
Fungi (mycosis)	*C. neoformans*	nIgM and antigen IgM protects against systemic dissemination. Important for IFN-γ response and activation of macrophages.	(121, 122)
	P. carinii	nIgM protects against dissemination and priming of TH2 and TH17 responses	(123)
	A. fumigatus	Anti-GlcNAc IgM antibodies protect against allergic asthma	(124, 125),
Viruses (viral infections)	*Influenza A*	sIgM-deficient mice show poor viral neutralizing ability and increased viral titers	(126–128),
	VSV	Natural IgM traps VSV antigens in secondary lymphoid tissues	(129–131),
	RVS	IgM BCR on Bregs a target for RVS and detrimental to disease	(132)
Bacteria	*S. pneumonia*	Adoptive transfer of B1a cells derived sIgM led to improved survival of infected μMT mice. sIgM was dependent on GM-CSF	(133)
	E. coli	Adoptive transfer of B1a cells derived sIgM led to improved survival of infected μMT mice. sIgM was dependent on GM-CSF	(133)
	Ehrlichia muris	Bone marrow derived IgM-secreting cells, AID independent provide protection	(32, 134),
	F. tularensis	sIgM was directed at the LPS fraction of *F. tularensis* and depended on IL-1β	(135)
	Haemophilus influenzae	PD-L2 dependent B 1 natural IgM anti-phosphorycholine provide protection against H. influenzae	(136)
Non-infectious agents	SLE	Autoantibodies IgM anti-dsDNA are protective, sIgM mice protected	(115, 137, 138),
	Allergy	Anti-GlcNAc IgM antibodies passively administered or vaccine induced protective	(23, 124),
	Cancer	Natural IgM recognized sugar moieties include MUCIN 1, SAM6, PAM-1 in cancerous cells	

SLE, systemic lupus erythematosus; VSV, vascular stomatis virus; nIgM, natural IgM; ABPA, allergic bronchopulmonary aspergillosis.

IgM memory cells). However, we mainly focus on discussing IgM contribution in regulating bacterial infections particularly in mucosal sites in experimental infection models (88). In the human gut mucosa, several studies have found human secreted IgM, together with secreted IgA, to coat human microbiota (88, 151, 152). IgM enhanced IgA binding repertoire and in some instances was even more potent in neutralizing enteric bacteria on its own (151). Specifically, IgM was found to promote bacterial species that are beneficial for healthy gut homeostasis, such as Firmicutes (e.g., *Bacillus cereus*, *Lachnospiraceae* spp. and *Ruthenibacterium* spp.) and Bacteroidetes (*Bacteroides vulgatus*) which are all beneficial (88, 146, 153). Age negatively correlated with the presence of these bacteria, resulting in dysbiosis in the adult population (153). Secreted IgM/MCBs-M may have developed to aid IgA in preserving microbiota homeostasis by directly interacting with bacteria to promote abundance of healthy microbiota and possibly eliminating pathogenic bacteria.

In the lung mucosa, infection of B cell deficient mice (μMT mice) with *Escherichia coli* or *Streptococcus pneumoniae* led to increased mortality and lung bacterial burdens (**Table 1**) (133). Transfer of wild type mice pleural cavity B1a cells, which secrete copious amounts of sIgM led to improved survival of infected μMT mice (133). Granulocyte-macrophage colony stimulating factor (GM-CSF) was found to be essential in sIgM B1a induced protection, as transfer of B1 cells lacking this cytokine did not rescue infected μMT mice (133). Induced sIgM produced by B1a cells has also been shown to be essential in *Francisella tularensis* infection (135). In this infection model, production of sIgM was directed at the LPS fraction of *F. tularensis* and depended on IL-1β for its earlier protective effects. Interestingly, sIgM showed great specificity to *F. tularensis* and did not cross-react with *E. coli* LPS, suggesting that it was induced sIgM, and not natural occurring sIgM (135).

Emerging evidence suggests a localized B cell repertoire in the lamina propria which can influence BM and peritoneal cavity B cell populations (9). Mono-colonization of germ-free mice influenced VDJ recombination process in the LP (9). In another study, neonatal immunization with group A streptococcus antigen increased GlcNAc reactive B cells and clonotype diversity in adult mice (10). These GlcNAc reactive B cells were educated in the LP in early life and disseminated systemically to provide protection against GlcNAc containing species (10). Early education of B cells might support diversification of the B cell repertoire but needs further investigations.

Immunoglobulin M Against Fungi

Natural IgM antibodies directed against fungal pathogens are important in both complement-dependent and -independent fungal recognition and clearance (154) and have been shown to have direct killing effects (155). Most natural IgM antibodies are conserved across species and are not dependent on antigen exposure, as suggested from their presence in germ-free mice and umbilical cord blood of non-human primates and humans (123). In fungi, these natural IgM antibodies are directed to conserved major cell wall components β-(1,3)-glucan and chitin and are derived from B1 cells in the mouse spleen (**Table 1**) (121, 123, 154, 156).

Mice deficient of sIgM show increased dissemination of *Cryptococcus neoformans* to other organs such as spleen, kidney, and brain when infected intravenously (121). In this setting, sIgM is thought to contribute to the optimal Th1 induction and the subsequent activation of phagocytic macrophages that kill the fungus (121). B cells, and more specifically IgM, were shown to be essential in protective mechanisms against *C. neoformans* when naïve B cells were transferred to RAG-1-deficient mice (121, 156). Transfer of B cells was shown to reduce fungal dissemination to the brain but had no effect in lung fungal burden (156). Both natural and infection induced-IgM were important in the control of *C. neoformans* and contributed to the optimal Th1 cytokine production (121, 156). A human study using antibodies generated against *C. neoformans* glucuronoxylomannan in a transgenic mouse expressing human IgM, revealed that protective effects of IgM were epitope specific and route of injection dependent (122). Non-protective effects of sIgM have been observed when sIgM-deficient mice were injected intraperitoneally, with increase in their survival compared to control wild type mice (157). In Pneumocystis, an opportunistic fungi that infects HIV/AIDS patients, natural IgM antibodies are detected and have an important role in clearance (158). Mice lacking sIgM are susceptible to pulmonary *Pneumocystis carinii* infection and show increased burdens, which are associated with altered inflammatory response (**Table 1**) (123). Secreted IgM deficiency in mice is associated with reduced IL-6 and IL-1β innate cytokine production and adaptive TH2 and TH17 responses at both lung and draining lymph nodes (123). The susceptibility of sIgM-deficient mice to *P. carinii* infection is likely to be due to defective DC presentation and priming of CD4 T cells and a lack of class switching to protective mucosal IgG and IgA isotypes (123). Individuals with X-linked hyper-IgM syndrome due to CD40L mutation, display equal susceptibility to pulmonary fungal infections, which may suggest a minimal role for antibodies in these infections (159, 160). In both experimental models and in humans where sIgM or B cell antibody function was blocked by anti-CD20 monoclonal antibodies, severe defects in optimal innate and adaptive responses occurred, resulting in susceptibility to fungal infections. This is suggestive of a critical function of natural IgM.

Immunoglobulin M Against Parasites

The role of antibodies in trypanosoma parasitic control are well documented, where a constant battle to opsonize and kill parasites occurs, while parasites have developed complex variant specific surface glycoproteins (VSGs) to avoid host recognition (**Figure 3**) (161, 162). *Trypanosoma evansi* can infect all domesticated animals and is transmitted by biting sand flies and vampire bats (163). Antibodies, particularly IgM isotype have been shown to be important in the control of *T. evansi* (120). Type 1 cytokines and effector molecules such as IFN-γ, TNF-α, and iNOS were found to be redundant in a mouse model of *T. evansi* infection. In contrast, mice deficient of IgM or B cells succumbed significantly quicker to *T. evansi* infection and were not able to control parasitemia (**Table 1**) (120). Furthermore, IgM, rather than IgG, was found to be critical in

parasitemia control as passive transfusion with *T. evansi* immune IgM serum, but not IgG serum protected naïve mice from re-infection with the same parasite (120). Complement, which kills parasites through phagocytosis *via* complement receptor mediated recognition, did not play a role in this instance, suggesting other mechanisms of parasite killing. In a pleomorphic *Trypanosoma brucei* AnTat 1.1E infection model, B cells and IgM were found to play minimal roles in trypanosomiasis associated anemia, parasite induced anti-VSG antibodies, host survival, and disease progression (119). Mice lacking IgM showed similar levels of parasitemia to wild type counterparts when infected intraperitoneally, exposed to tsetse fly bites or non-virulent field isolates (119). Similarly, to *T. evansi* infections, in IgM-deficient infected mice, an increase in VSG specific-IgD isotype antibody production was observed, as well as normal levels of VSG specific-IgG2a or IgG3, which are thought to have compensated for the loss of IgM (129). Interestingly, B cells which are thought to induce immune pressure in pleomorphic *T. brucei* were found to be redundant in this instance and VSG intergenic switching occurred independently of antibody or IgM presence (119). A recent study showed an important function of natural and induced IgM antibodies against trypanosome lytic factors (TLF2) in *T. brucei* infected people (162). Healthy people were found to harbor germline encoded natural IgM antibodies against TLF2, which were further upregulated by *T. brucei rhodesiense* infection and reduced by treatment with suramin or melarsoprol (162). TLF2-IgMs antibodies interact with the TLF protein, haptoglobin related protein (HPR), thus offering a route for parasite endocytosis and killing *via* alternative complement activation (162).

IgM antibodies specific to α-gal have been shown to be protective against *Plasmodium falciparum*, a malaria causing parasite (117). IgM antibodies against α-gal are thought to be generated in the gut by microbiota that express α-gal, such as certain strains of *E. coli* (O86:B7) (23). In human, anti-α-gal IgM antibodies can directly bind to *P. falciparum* sporozoite and initiate complement activation and parasite clearance (117). Children between 0 and 1 years old in malaria endemic areas are at the highest risk of developing the disease, which is associated with reduced anti-α-gal IgM antibodies in serum. In older children the level of anti-α-gal IgM antibodies increases, associated with added protection from malaria parasite and this is partly attributed to the maturity of the B cell compartment. These anti-α-gal IgM antibodies were induced in germ-free animals mono-colonized with *E. coli* (O86:B7) strain and were found to be protective when these mice were infected with different malaria parasites (117). Interestingly, these anti-α-gal IgM antibodies did not depend on AID, suggesting that these were natural IgM antibodies generated outside germinal centers and did not undergo somatic hypermutation (117). Other natural IgM memory B cells able to recognize merozoite surface protein 1 (MSP1) protein of *P. falciparum* have been shown to be considerably more rapid than IgG and confer protection against re-challenge with the parasite (164). Similarly, to anti-α-gal IgM antibodies, these anti-MSP IgM B cells gave rise to mainly T cell-independent high affinity plasma cells ($B220^+CD138^+$) and T cell-dependent ($B220^-CD138^+$) IgM plasma cells (164). These IgM memory B cells produce IgM plasma cells with similar binding affinity to class switched IgG plasma cells (164). It is plausible to assume that these memory IgM B cells developed as a strategy to protect against primary and secondary Plasmodium infection to prevent dissemination of parasites pre-GCs B cells, capable of generating high affinity IgG plasma cells.

Immunoglobulin M Against Viruses

Early control of viral infections is dependent on innate natural antibodies and most vaccine strategies target potent neutralizing antibodies. Natural IgM antibodies can bind to surface glycoproteins of most viral capsids and activate the complement system *via* classical pathways, leading to viral opsonization and killing (126). Influenza virus is a rapidly replicating respiratory virus that is detected by natural IgM antibodies, which do not require AID or class switch recombination or somatic hypermutated B cells (93). In the absence of adaptive immune cells, including B and T cells, such as in the case of severe combined immunodeficiency (SCID), influenza virus is uncontrollable and causes death in animals (127, 165). Mice lacking sIgM are susceptible to influenza virus and show poor viral neutralizing ability leading to increased viral titers (128). Adoptively transfer of naïve or influenza primed serum to sIgM-deficient or RAG-1-deficient mice restores viral neutralizing ability and virus clearance (**Table 1**) (128). Vesicular stomatitis virus (VSV), an enveloped RNA virus requires both natural IgM and complement for clearance (130). Human sera lacking any of the early complement factors C1–C5, but not late complement factors C6–C9 is unable to kill VSV infected cells. These complement factors rely on natural IgM presence on sera for effective killing of VSV infected cells (130). Interestingly, mice contain natural VSV IgM antibodies that were induced independently of infection (131). These antibodies were essential in limiting early VSV dissemination to vital organs, such as the kidney, brain, and lungs and neutralized the virus in secondary lymphoid tissues (131). In IgM-deficient mice or B cell-deficient mice, VSV was recruited to secondary lymphoid tissue, where it accumulated and activated the natural IgM antibody response (**Table 1**). This, in turn, delayed dissemination of VSV to the kidneys and brain and allowed activation of the adaptive immune response, thus reducing VSV titers at early time points in these tissues (131). Furthermore, IgM-deficient mice show a delayed antibody class switching to neutralizing IgG, which illuminated this trapping of VSV antigens in secondary lymphoid tissues by natural IgM (129, 131).

CONCLUDING REMARKS

Classic memory takes up to 4 days to develop and may be slow relative to the rapid invasion of encapsulated bacteria and viruses. It is during this period that innate-like B cells, which produce rapid cross-reactive natural IgM or long-lasting antigen-specific IgM responses that can interfere with initial infection. As cross-reactive SLPC, they can assist phagocytes

and complement, to clear the system and mucosal sites. Antigen-specific LLPC can rapidly class switch to specific isotype or become highly specific IgM producing cells able to clear infection or activate other adaptive cells. However, despite all this knowledge, little attention has been paid to their role in immune responses or how their production can be manipulated to the host's advantage. The higher diversity of MBCs provides an evolutionary advantage to the host, where there is increased antibody breadth protection, a phenomenon that is critical in most antibody-based vaccine designs. We do not fully understand the role of IgM in allergies beyond class switching and its role in lung mucosal sites where it has been suggested that it can be hijacked by viruses to gain entry in mucosal sites. Whether natural or induced IgM can be fine-tuned to fight cancers and other infections is an area still less explored.

AUTHOR CONTRIBUTIONS

SH conceived the idea. KJ, AS, FB, and SH wrote the paper. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Akula S, Mohammadamin S, Hellman L. Fc Receptors for Immunoglobulins and Their Appearance during Vertebrate Evolution. *PloS One* (2014) 9(5): e96903. doi: 10.1371/journal.pone.0096903
2. Xu Z, Parra D, Gómez D, Salinas I, Zhang Y-A, von Gersdorff Jørgensen L, et al. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proc Natl Acad Sci* (2013) 110(32):13097–102. doi: 10.1073/pnas.1304319110
3. Blandino R, Baumgarth N. Secreted IgM: New tricks for an old molecule. *J Leukoc Biol* (2019) 106(5):1021–34. doi: 10.1002/JLB.3RI0519-161R
4. Baumgarth N, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 Cell–Derived Immunoglobulin M Antibodies Are Nonredundant Components of the Protective Response to Influenza Virus Infection. *J Exp Med* (2000) 192(2):271–80. doi: 10.1084/jem.192.2.271
5. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* (2010) 10(11):778–86. doi: 10.1038/nri2849
6. Michaud E, Mastrandrea C, Rochereau N, Paul S. Human Secretory IgM: An Elusive Player in Mucosal Immunity. *Trends Immunol* (2020) 41(2):141–56. doi: 10.1016/j.it.2019.12.005
7. Quartier P, Potter PK, Ehrenstein MR, Walport MJ, Botto M. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. *Eur J Immunol* (2005) 35(1):252–60. doi: 10.1002/eji.200425497
8. Litvack ML, Post M, Palaniyar N. IgM Promotes the Clearance of Small Particles and Apoptotic Microparticles by Macrophages. *PloS One* (2011) 6 (3):e17223. doi: 10.1371/journal.pone.0017223
9. Wesemann DR, Portuguese AJ, Meyers RM, Gallagher MP, Cluff-Jones K, Magee JM, et al. Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature* (2013) 501(7465):112–5. doi: 10.1038/nature12496
10. New JS, Dizon BLP, Fucile CF, Rosenberg AF, Kearney JF, King RG. Neonatal Exposure to Commensal-Bacteria-Derived Antigens Directs Polysaccharide-Specific B-1 B Cell Repertoire Development. *Immunity* (2020) 53(1):172–186.e6. doi: 10.1016/j.immuni.2020.06.006
11. Kawahara T, Ohdan H, Zhao G, Yang Y-G, Sykes M. Peritoneal Cavity B Cells Are Precursors of Splenic IgM Natural Antibody-Producing Cells. *J Immunol* (2003) 171(10):5406–14. doi: 10.4049/jimmunol.171.10.5406
12. Nguyen TTT, Graf BA, Randall TD, Baumgarth N. sIgM–FcμR Interactions Regulate Early B Cell Activation and Plasma Cell Development after Influenza Virus Infection. *J Immunol* (2017) 199(5):1635–46. doi: 10.4049/jimmunol.1700560
13. Hobbs JR, Milner RD, Watt PJ. Gamma-M deficiency predisposing to meningococcal septicaemia. *Br Med J* (1967) 4(5579):583–6. doi: 10.1136/bmj.4.5579.583
14. Kaufman H, Hobbs J. Immunoglobulin Deficiencies In An Atopic Population. *Lancet* (1970) 296(7682):1061–3. doi: 10.1016/S0140-6736(70)90288-6
15. Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, et al. Positive Selection of Natural Autoreactive B Cells. *Science (80-)* (1999) 285 (5424):113–6. doi: 10.1126/science.285.5424.113
16. Hastings WD, Gurdak SM, Tumang JR, Rothstein TL. CD5+/Mac-1–peritoneal B cells: A novel B cell subset that exhibits characteristics of B-1 cells. *Immunol Lett* (2006) 105(1):90–6. doi: 10.1016/j.imlet.2006.01.002
17. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell–specified progenitor. *Nat Immunol* (2006) 7(3):293–301. doi: 10.1038/ni1301
18. Montecino-Rodriguez E, Dorshkind K. B-1 B Cell Development in the Fetus and Adult. *Immunity* (2012) 36(1):13–21. doi: 10.1016/j.immuni.2011.11.017
19. Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, Porayette P, Shelley WC, Conway SJ, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci* (2011) 108(4):1468–73. doi: 10.1073/pnas.1015841108
20. Hayakawa K, Hardy RR, Stall AM, Herzenberg LA, Herzenberg LA. Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. *Eur J Immunol* (1986) 16(10):1313–6. doi: 10.1002/eji.1830161021
21. Ghosn EEB, Sadate-Ngatchou P, Yang Y, Herzenberg LA, Herzenberg LA. Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen. *Proc Natl Acad Sci* (2011) 108(7):2879–84. doi: 10.1073/pnas.1019764108
22. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol* (2012) 42(1):120–9. doi: 10.1002/eji.201141890
23. Kearney JF, Patel P, Stefanov EK, King RG. Natural Antibody Repertoires: Development and Functional Role in Inhibiting Allergic Airway Disease. *Annu Rev Immunol* (2015) 33(1):475–504. doi: 10.1146/annurev-immunol-032713-120140
24. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) 11(1):34–46. doi: 10.1038/nri2901
25. Hardy RR, Hayakawa K. B Cell Development Pathways. *Annu Rev Immunol* (2001) 19(1):595–621. doi: 10.1146/annurev.immunol.19.1.595
26. Smith FL, Baumgarth N. B-1 cell responses to infections. *Curr Opin Immunol* (2019) 57:23–31. doi: 10.1016/j.coi.2018.12.001
27. Ansel KM, Harris RBS, Cyster JG. CXCL13 Is Required for B1 Cell Homing, Natural Antibody Production, and Body Cavity Immunity. *Immunity* (2002) 16(1):67–76. doi: 10.1016/S1074-7613(01)00257-6
28. Yang Y, Wang C, Yang Q, Kantor AB, Chu H, Ghosn EEB, et al. Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *Elife* (2015) 4:e09083. doi: 10.7554/eLife.09083
29. Yang Y, Tung JW, Ghosn EEB, Herzenberg LA, Herzenberg LA. Division and differentiation of natural antibody-producing cells in mouse spleen. *Proc Natl Acad Sci* (2007) 104(11):4542–46. doi: 10.1073/pnas.0700001104
30. Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. *Proc Natl Acad Sci* (1992) 89(8):3320–24. doi: 10.1073/pnas.89.8.3320

31. Bhat NM, Kantor AB, Bieber MM, Stall AM, Herzenberg LA, Teng NNH. The ontogeny and functional characteristics of human B-1 (CD5+ B) cells. *Int Immunol* (1992) 4(2):243–52. doi: 10.1093/intimm/4.2.243
32. Racine R, McLaughlin M, Jones DD, Wittmer ST, MacNamara KC, Woodland DL, et al. IgM Production by Bone Marrow Plasmablasts Contributes to Long-Term Protection against Intracellular Bacterial Infection. *J Immunol* (2011) 186(2):1011–21. doi: 10.4049/jimmunol.1002836
33. McLean KC, Mandal M. It Takes Three Receptors to Raise a B Cell. *Trends Immunol* (2020) 41(7):629–42. doi: 10.1016/j.it.2020.05.003
34. Clark MR, Mandal M, Ochiai K, Singh H. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nat Rev Immunol* (2014) 14(2):69–80. doi: 10.1038/nri3570
35. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat Rev Immunol* (2009) 9(3):195–205. doi: 10.1038/nri2491
36. Küppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* (2005) 5(4):251–62. doi: 10.1038/nrc1589
37. Fistonich C, Zehentmeier S, Bednarski JJ, Miao R, Schjerven H, Sleckman BP, et al. Cell circuits between B cell progenitors and IL-7+ mesenchymal progenitor cells control B cell development. *J Exp Med* (2018) 215(10):2586–99. doi: 10.1084/jem.20180778
38. Mandal M, Okoreeh MK, Kennedy DE, Maienschein-Cline M, Ai J, McLean KC, et al. CXCR4 signaling directs Igk recombination and the molecular mechanisms of late B lymphopoiesis. *Nat Immunol* (2019) 20(10):1393–403. doi: 10.1038/s41590-019-0468-0
39. Köhler F, Hug E, Eschbach C, Meixlsperger S, Hobeika E, Kofer J, et al. Autoreactive B Cell Receptors Mimic Autonomous Pre-B Cell Receptor Signaling and Induce Proliferation of Early B Cells. *Immunity* (2008) 29 (6):912–21. doi: 10.1016/j.immuni.2008.10.013
40. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science (80-)* (1990) 248(4962):1517–23. doi: 10.1126/science.2360047
41. Tokoyoda K, Egawa T, Sugiyama T, Choi B-I, Nagasawa T. Cellular Niches Controlling B Lymphocyte Behavior within Bone Marrow during Development. *Immunity* (2004) 20(6):707–18. doi: 10.1016/j.immuni.2004.05.001
42. Sabouri Z, Perotti S, Spierings E, Humburg P, Yabas M, Bergmann H, et al. IgD attenuates the IgM-induced anergy response in transitional and mature B cells. *Nat Commun* (2016) 7(1):13381. doi: 10.1038/ncomms 13381
43. Übelhart R, Hug E, Bach MP, Wossning T, Dühren-von Minden M, Horn AHC, et al. Responsiveness of B cells is regulated by the hinge region of IgD. *Nat Immunol* (2015) 16(5):534–43. doi: 10.1038/ni.3141
44. Noviski M, Mueller JL, Satterthwaite A, Garrett-Sinha LA, Brombacher F, Zikherman J, et al. IgM and IgD B cell receptors differentially respond to endogenous antigens and control B cell fate. *Elife* (2018) 7:e35074. doi: 10.7554/eLife.35074
45. Gauld SB, Benschop RJ, Merrell KT, Cambier JC. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* (2005) 6(11):1160–7. doi: 10.1038/ni1256
46. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol* (2007) 7 (8):633–43. doi: 10.1038/nri2133
47. Victora GD, Nussenzweig MC. Germinal Centers. *Annu Rev Immunol* (2012) 30(1):429–57. doi: 10.1146/annurev-immunol-020711-075032
48. Stewart I, Radtke D, Phillips B, McGowan SJ, Bannard O. Germinal Center B Cells Replace Their Antigen Receptors in Dark Zones and Fail Light Zone Entry when Immunoglobulin Gene Mutations are Damaging. *Immunity* (2018) 49(3):477–89.e7. doi: 10.1016/j.immuni.2018.08.025
49. Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, et al. Germinal Center B Cell and T Follicular Helper Cell Development Initiates in the Interfollicular Zone. *Immunity* (2011) 34(6):947–60. doi: 10.1016/j.immuni.2011.03.024
50. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (AID), a Potential RNA Editing Enzyme. *Cell* (2000) 102(5):553–63. doi: 10.1016/S0092-8674(00)00078-7
51. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, et al. Activation-Induced Cytidine Deaminase (AID) Deficiency Causes the Autosomal Recessive Form of the Hyper-IgM Syndrome (HIGM2). *Cell* (2000) 102(5):565–75. doi: 10.1016/S0092-8674(00)00079-9
52. Alt FW, Zhang Y, Meng F, Guo C, Schwer B. Review Mechanisms of Programmed DNA Lesions and Genomic Instability in the Immune System. *Cell* (2013) 152(3):417–29. doi: 10.1016/j.cell.2013.01.007
53. Shinall SM, Gonzalez-Fernandez M, Noelle RJ, Waldschmidt TJ. Identification of Murine Germinal Center B Cell Subsets Defined by the Expression of Surface Isotypes and Differentiation Antigens. *J Immunol* (2000) 164(11):5729–38. doi: 10.4049/jimmunol.164.11.5729
54. Wilson M, Hsu E, Marcuz A, Courtet M, Du Pasquier L, Steinberg C. What limits affinity maturation of antibodies in Xenopus–the rate of somatic mutation or the ability to select mutants? *EMBO J* (1992) 11(12):4337–47. doi: 10.1002/j.1460-2075.1992.tb05533.x
55. Hinds-Frey KR, Nishikata H, Litman RT, Litman GW. Somatic variation precedes extensive diversification of germline sequences and combinatorial joining in the evolution of immunoglobulin heavy chain diversity. *J Exp Med* (1993) 178(3):815–24. doi: 10.1084/jem.178.3.815
56. Le Gallou S, Zhou Z, Thai L-H, Fritzen R, de Los Aires AV, Mégret J, et al. A splenic IgM memory subset with antibacterial specificities is sustained from persistent mucosal responses. *J Exp Med* (2018) 215(8):2035–53. doi: 10.1084/jem.20180977
57. Bohannon C, Powers R, Satyabhama L, Cui A, Tipton C, Michaeli M, et al. Long-lived antigen-induced IgM plasma cells demonstrate somatic mutations and contribute to long-term protection. *Nat Commun* (2016) 7 (1):11826. doi: 10.1038/ncomms11826
58. Thierry GR, Kuka M, De Giovanni M, Mondor I, Brouilly N, Iannacone M, et al. The conduit system exports locally secreted IgM from lymph nodes. *J Exp Med* (2018) 215(12):2972–83. doi: 10.1084/jem.20180344
59. Sørensen V, Rasmussen IB, Sundvold V, Michaelsen TE, Sandlie I. Structural requirements for incorporation of J chain into human IgM and IgA. *Int Immunol* (2000) 12(1):19–27. doi: 10.1093/intimm/12.1.19
60. Norderhaug IN, Johansen F-E, Krajči P, Brandtzaeg P. Domain deletions in the human polymeric Ig receptor disclose differences between its dimeric IgA and pentameric IgM interaction. *Eur J Immunol* (1999) 29(10):3401–9. doi: 10.1002/(SICI)1521-4141(199910)29:10<3401::AID-IMMU3401>3.0.CO;2-G
61. Johansen F-E, Pekna M, Norderhaug IN, Haneberg B, Hietala MA, Krajci P, et al. Absence of Epithelial Immunoglobulin a Transport, with Increased Mucosal Leakiness, in Polymeric Immunoglobulin Receptor/Secretory Component–Deficient Mice. *J Exp Med* (1999) 190(7):915–22. doi: 10.1084/jem.190.7.915
62. Woof JM, Mestecky J. Mucosal immunoglobulins. *Immunol Rev* (2005) 206 (1):64–82. doi: 10.1111/j.0105-2896.2005.00290.x
63. Brewer JW, Randall TD, Parkhouse RM, Corley RB. Mechanism and subcellular localization of secretory IgM polymer assembly. *J Biol Chem* (1994) 269(25):17338–48.
64. Czajkowsky DM, Shao Z. The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. *Proc Natl Acad Sci* (2009) 106(35):14960–5. doi: 10.1073/pnas.0903805106
65. Hombach J, Tsubata T, Leclercq L, Stappert H, Reth M. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature* (1990) 343(6260):760–2. doi: 10.1038/343760a0
66. Davis AC, Roux KH, Shulman MJ. On the structure of polymeric IgM. *Eur J Immunol* (1988) 18(7):1001–8. doi: 10.1002/eji.1830180705
67. Hurst MM, Volanakis JE, Stroud RM, Bennett JC. C1 fixation and classical complement pathway activation by a fragment of the Cmu4 domain of IgM. *J Exp Med* (1975) 142(5):1322–6. doi: 10.1084/jem.142.5.1322
68. Sharp TH, Boyle AL, Diebolder CA, Kros A, Koster AJ, Gros P. Insights into IgM-mediated complement activation based on in situ structures of IgM-C1-C4b. *Proc Natl Acad Sci* (2019) 116(24):11900–5. doi: 10.1073/pnas.1901841116
69. Srinivasan L, Sasaki Y, Calado DP, Zhang B, Paik JH, DePinho RA, et al. PI3 Kinase Signals BCR-Dependent Mature B Cell Survival. *Cell* (2009) 139 (3):573–86. doi: 10.1016/j.cell.2009.08.041
70. Pillai S, Cariappa A, Moran ST. Positive selection and lineage commitment during peripheral B-lymphocyte development. *Immunol Rev* (2004) 197 (1):206–18. doi: 10.1111/j.0105-2896.2003.097.x
71. Lam K-P, Kühn R, Rajewsky K. In Vivo Ablation of Surface Immunoglobulin on Mature B Cells by Inducible Gene Targeting Results in Rapid Cell Death. *Cell* (1997) 90(6):1073–83. doi: 10.1016/S0092-8674(00)80373-6

72. Wiersma EJ, Chen F, Bazin R, Collins C, Painter RH, Lemieux R, et al. Analysis of IgM structures involved in J chain incorporation. *J Immunol* (1997) 158(4):1719–26.
73. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA, et al. Human Serum IgM Glycosylation: IDENTIFICATION OF GLYCOFORMS THAT CAN BIND TO MANNAN-BINDING LECTIN. *J Biol Chem* (2005) 280 (32):29080–7. doi: 10.1074/jbc.M504528200
74. Giannone C, Fagioli C, Valetti C, Sitia R, Anelli T. Roles of N-glycans in the polymerization-dependent aggregation of mutant Ig-μ chains in the early secretory pathway. *Sci Rep* (2017) 7(1):41815. doi: 10.1038/srep41815
75. Carroll MC. The Role Of Complement And Complement Receptors In Induction And Regulation Of Immunity. *Annu Rev Immunol* (1998) 16 (1):545–68. doi: 10.1146/annurev.immunol.16.1.545
76. Honjo K, Kubagawa Y, Jones DM, Dizon B, Zhu Z, Ohno H, et al. Altered Ig levels and antibody responses in mice deficient for the Fc receptor for IgM (FcμR). *Proc Natl Acad Sci* (2012) 109(39):15882–7. doi: 10.1073/pnas.1206567109
77. Kubagawa H, Oka S, Kubagawa Y, Torii I, Takayama E, Kang D-W, et al. Identity of the elusive IgM Fc receptor (FcμR) in humans. *J Exp Med* (2009) 206(12):2779–93. doi: 10.1084/jem.20091107
78. Nguyen TTT, Kläsener K, Zürn C, Castillo PA, Brust-Mascher I, Imai DM, et al. The IgM receptor FcμR limits tonic BCR signaling by regulating expression of the IgM BCR. *Nat Immunol* (2017) 18(3):321–33. doi: 10.1038/ni.3677
79. Uren TK, Johansen F-E, Wijburg OLC, Koentgen F, Brandtzaeg P, Strugnell RA. Role of the Polymeric Ig Receptor in Mucosal B Cell Homeostasis. *J Immunol* (2003) 170(5):2531–9. doi: 10.4049/jimmunol.170.5.2531
80. Rochereau N, Drocourt D, Perouzel E, Pavot V, Redelinghuys P, Brown GD, et al. Dectin-1 Is Essential for Reverse Transcytosis of Glycosylated SIgA-Antigen Complexes by Intestinal M Cells. *PloS Biol* (2013) 11(9):e1001658. doi: 10.1371/journal.pbio.1001658
81. Shibuya A, Sakamoto N, Shimizu Y, Shibuya K, Osawa M, Hiroyama T, et al. Fcα/μ receptor mediates endocytosis of IgM-coated microbes. *Nat Immunol* (2000) 1(5):441–6. doi: 10.1038/80886
82. Shibuya A, Honda S, Shibuya K. A pro-inflammatory role of Fcα/μR on marginal zone B cells in sepsis. *Int Immunol* (2017) 29(11):519–24. doi: 10.1093/intimm/dxx059
83. Wu YL, Stubbington MJT, Daly M, Teichmann SA, Rada C. Intrinsic transcriptional heterogeneity in B cells controls early class switching to IgE. *J Exp Med* (2016) 214(1):183–96. doi: 10.1084/jem.20161056
84. Zhang X, Zhang Y, Ba Z, Kyritsis N, Casellas R, Alt FW. Fundamental roles of chromatin loop extrusion in antibody class switching. *Nature* (2019) 575 (7782):385–9. doi: 10.1038/s41586-019-1723-0
85. Stavnezer J, Schrader CE. IgH Chain Class Switch Recombination: Mechanism and Regulation. *J Immunol* (2014) 193(11):5370–8. doi: 10.4049/jimmunol.1401849
86. Siebenkotten G, Esser C, Radbruch A, Wabl M. The murine IgG1/IgE class switch program. *Eur J Immunol* (1992) 22(7):1827–34. doi: 10.1002/eji.1830220723
87. Alt FW, Blackwell TK, Yancopoulos GD. Development of the primary antibody repertoire. *Science (80-)* (1987) 238(4830):1079–87. doi: 10.1126/science.3317825
88. Magri G, Comerma L, Pybus M, Sintes J, Lligé D, Segura-Garzón D, et al. Human Secretory IgM Emerges from Plasma Cells Clonally Related to Gut Memory B Cells and Targets Highly Diverse Commensals. *Immunity* (2017) 47(1):118–134.e8. doi: 10.1016/j.immuni.2017.06.013
89. Wesemann DR, Magee JM, Boboila C, Calado DP, Gallagher MP, Portuguese AJ, et al. Immature B cells preferentially switch to IgE with increased direct S u to S e recombination. *J Exp Med* (2011) 208:(13):2733–46. doi: 10.1084/jem.20111155
90. Hara Y, Tashiro Y, Murakami A, Nishimura M, Shimizu T, Kubo M, et al. High affinity IgM+ memory B cells are generated through a germinal center-dependent pathway. *Mol Immunol* (2015) 68(2, Part C):617–27. doi: 10.1016/j.molimm.2015.10.003
91. Seifert M, Przekopowitz M, Taudien S, Lollies A, Ronge V, Drees B, et al. Functional capacities of human IgM memory B cells in early inflammatory responses and secondary germinal center reactions. *Proc Natl Acad Sci* (2015) 112(6):E546–55. doi: 10.1073/pnas.1416276112
92. Tashiro Y, Murakami A, Hara Y, Shimizu T, Kubo M, Goitsuka R, et al. High-affinity IgM+ memory B cells are defective in differentiation into IgM antibody-secreting cells by re-stimulation with a T cell-dependent antigen. *Sci Rep* (2018) 8(1):14559. doi: 10.1038/s41598-018-32926-w
93. Harada Y, Muramatsu M, Shibata T, Honjo T, Kuroda K. Unmutated Immunoglobulin M Can Protect Mice from Death by Influenza Virus Infection. *J Exp Med* (2003) 197(12):1779–85. doi: 10.1084/jem.20021457
94. Tarlinton D, Good-Jacobson K. Diversity Among Memory B Cells: Origin, Consequences, and Utility. *Science (80-)* (2013) 341(6151):1205–11. doi: 10.1126/science.1241146
95. Pape KA, Maul RW, Dileepan T, Paustian AS, Gearhart PJ, Jenkins MK. Naive B Cells with High-Avidity Germline-Encoded Antigen Receptors Produce Persistent IgM^+ and Transient IgG^+ Memory B Cells. *Immunity* (2018) 48(6):1135–43.e4. doi: 10.1016/j.immuni.2018.04.019
96. Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med* (2006) 203 (4):1081–91. doi: 10.1084/jem.20060087
97. Sintes J, Gentile M, Zhang S, Garcia-Carmona, Y, Magri G, Cassis L, et al. mTOR intersects antibody-inducing signals from TACI in marginal zone B cells. *Nat Commun* (2017) 8(1):1462. doi: 10.1038/s41467-017-01602-4
98. Taylor JJ, Pape KA, Jenkins MK. A germinal center–independent pathway generates unswitched memory B cells early in the primary response. *J Exp Med* (2012) 209(3):597–606. doi: 10.1084/jem.20111696
99. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B Cell Populations Mediate Early and Late Memory During an Endogenous Immune Response. *Science (80-)* (2011) 331(6021):1203–07. doi: 10.1126/science.1201730
100. Dogan I, Bertocci B, Vilmont V, Delbos F, Mégret J, Storck S, et al. Multiple layers of B cell memory with different effector functions. *Nat Immunol* (2009) 10(12):1292–9. doi: 10.1038/ni.1814
101. Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med* (2005) 201(4):545–54. doi: 10.1084/jem.20042060
102. Mesin L, Schiepers A, Ersching J, Barbulescu A, Cavazzoni CB, Angelini A, et al. Restricted Clonality and Limited Germinal Center Reentry Characterize Memory B Cell Reactivation by Boosting. *Cell* (2020) 180(1):92–106.e11. doi: 10.1016/j.cell.2019.11.032
103. Burton BR, Tennant RK, Love J, Titball RW, Wraith DC, White HN. Variant proteins stimulate more IgM+ GC B-cells revealing a mechanism of cross-reactive recognition by antibody memory. *Elife* (2018) 7:e26832. doi: 10.7554/eLife.26832
104. Good KL, Tangye SG. Decreased expression of Krüppel-like factors in memory B cells induces the rapid response typical of secondary antibody responses. *Proc Natl Acad Sci* (2007) 104(33):13420–5. doi: 10.1073/pnas.0703872104
105. Lam WY, Jash A, Yao CH, D'Souza L, Wong R, Nunley RM, et al. Metabolic and Transcriptional Modules Independently Diversify Plasma Cell Lifespan and Function. *Cell Rep* (2018) 24(9):2479–2492.e6. doi: 10.1016/j.celrep.2018.07.084
106. Weisel FJ, Mullett SJ, Elsner RA, Menk AV, Trivedi N, Luo W, et al. Germinal center B cells selectively oxidize fatty acids for energy while conducting minimal glycolysis. *Nat Immunol* (2020) 21(3):331–42. doi: 10.1038/s41590-020-0598-4
107. Shimizu T, Kozono Y, Kozono H, Oda M, Azuma T. Affinity maturation of secreted IgM pentamers on B cells. *Int Immunol* (2004) 16(5):675–84. doi: 10.1093/intimm/dxh069
108. Randen I, Brown D, Thompson KM, Hughes-Jones N, Pascual V, Victor K, et al. Clonally related IgM rheumatoid factors undergo affinity maturation in the rheumatoid synovial tissue. *J Immunol* (1992) 148(10):3296 LP – 3301.
109. Roco JA, Mesin L, Binder SC, Nefzger C, Gonzalez-Figueroa P, Canete PF, et al. Class-Switch Recombination Occurs Infrequently in Germinal Centers. *Immunity* (2019) 51(2):337–350.e7. doi: 10.1016/j.immuni.2019.07.001
110. Kato Y, Abbott RK, Freeman BL, Haupt S, Groschel B, Silva M, et al. Multifaceted Effects of Antigen Valency on B Cell Response Composition and Differentiation In Vivo. *Immunity* (2020) 53(3):548–63.e8. doi: 10.1016/j.immuni.2020.08.001

111. Klein U, Rajewsky K, Küppers R. Human Immunoglobulin (Ig)M+IgD+ Peripheral Blood B Cells Expressing the CD27 Cell Surface Antigen Carry Somatically Mutated Variable Region Genes: CD27 as a General Marker for Somatically Mutated (Memory) B Cells. *J Exp Med* (1998) 188(9):1679–89. doi: 10.1084/jem.188.9.1679
112. Rollenske T, Szijarto V, Lukasiewicz J, Guachalla LM, Stojkovic K, Hartl K, et al. Cross-specificity of protective human antibodies against Klebsiella pneumoniae LPS O-antigen. *Nat Immunol* (2018) 19(6):617–24. doi: 10.1038/s41590-018-0106-2
113. Lindner JM, Cornacchione V, Sathe A, Be C, Srinivas H, Riquet E, et al. Human Memory B Cells Harbor Diverse Cross-Neutralizing Antibodies against BK and JC Polyomaviruses. *Immunity* (2019) 50(3):668–676.e5. doi: 10.1016/j.immuni.2019.02.003
114. Weisel F, Shlomchik M. Memory B Cells of Mice and Humans. *Annu Rev Immunol* (2017) 35(1):255–84. doi: 10.1146/annurev-immunol-041015-055531
115. Witte T. IgM Antibodies Against dsDNA in SLE. *Clin Rev Allergy Immunol* (2008) 34(3):345–7. doi: 10.1007/s12016-007-8046-x
116. Reddy V, Martinez L, Isenberg DA, Leandro MJ, Cambridge G. Pragmatic Treatment of Patients With Systemic Lupus Erythematosus With Rituximab: Long-Term Effects on Serum Immunoglobulins. *Arthritis Care Res (Hoboken)* (2017) 69(6):857–66. doi: 10.1002/acr.22993
117. Yilmaz B, Portugal S, Tran TM, Gozzelino R, Ramos S, Gomes J, et al. Gut Microbiota Elicits a Protective Immune Response against Malaria Transmission. *Cell* (2014) 159(6):1277–89. doi: 10.1016/j.cell.2014.10.053
118. Pietrzak HM, Ioannidis LJ, Hansen DS. IgM+ memory B cells induced in response to Plasmodium berghei adopt a germinal centre B cell phenotype during secondary infection. *Parasitology* (2020) 147(9):994–8. doi: 10.1017/S003118202000061X
119. Magez S, Schwegmann A, Atkinson R, Claes F, Drennan M, De Baetselier P, et al. The Role of B-cells and IgM Antibodies in Parasitemia, Anemia, and VSG Switching in Trypanosoma brucei–Infected Mice. *PloS Pathog* (2008) 4 (8):e1000122. doi: 10.1371/journal.ppat.1000122
120. Baral TN, De Baetselier P, Brombacher F, Magez S. Control of Trypanosoma evansi Infection Is IgM Mediated and Does Not Require a Type I Inflammatory Response. *J Clin Oncol* (2007) 195:1513–20. doi: 10.1086/515577
121. Subramaniam KS, Datta K, Quintero E, Manix C, Marks MS, Pirofski L-A. The absence of serum IgM enhances the susceptibility of mice to pulmonary challenge with Cryptococcus neoformans. *J Immunol* (2010) 184(10):5755–67. doi: 10.4049/jimmunol.0901638
122. Maitta RW, Datta K, Chang Q, Luo RX, Witover B, Subramaniam K, et al. Protective and Nonprotective Human Immunoglobulin M Monoclonal Antibodies to Cryptococcus neoformans Glucuronoxylomannan Manifest Different Specificities and Gene Use Profiles. *Infect Immun* (2004) 72 (8):4810–8. doi: 10.1128/IAI.72.8.4810-4818.2004
123. Rapaka RR, Ricks DM, Alcorn JF, Chen K, Khader SA, Zheng M, et al. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina. *J Exp Med* (2010) 207(13):2907–19. doi: 10.1084/jem.20100034
124. Kin NW, Stefanov EK, Dizon BLP, Kearney JF. Antibodies Generated against Conserved Antigens Expressed by Bacteria and Allergen-Bearing Fungi Suppress Airway Disease. *J Immunol* (2012) 189(5):2246–56. doi: 10.4049/jimmunol.1200702
125. Ghosh S, Hoselton SA, Schuh JM. μ-Chain–Deficient Mice Possess B-1 Cells and Produce IgG and IgE, but Not IgA, following Systemic Sensitization and Inhalational Challenge in a Fungal Asthma Model. *J Immunol* (2012) 189 (3):1322–9. doi: 10.4049/jimmunol.1200138
126. Beebe DP, Schreiber RD, Cooper NR. Neutralization of influenza virus by normal human sera: mechanisms involving antibody and complement. *J Immunol* (1983) 130(3):1317–22. doi: 10.4049/jimmunol.1002103
127. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med* (2008) 205(13):3053–64. doi: 10.1084/jem.20080979
128. Jayasekera JP, Moseman EA, Carroll MC. Natural Antibody and Complement Mediate Neutralization of Influenza Virus in the Absence of Prior Immunity. *J Virol* (2007) 81(7):3487–94. doi: 10.1128/JVI.02128-06
129. Lutz C, Ledermann B, Kosco-Vilbois MH, Ochsenbein AF, Zinkernagel RM, Kohler G, et al. IgD can largely substitute for loss of IgM function in B cells. *Nature* (1998) 393(6687):797–801. doi: 10.1038/31716
130. Beebe DP, Cooper NR. Neutralization of vesicular stomatitis virus (VSV) by human complement requires a natural IgM antibody present in human serum. *J Immunol* (1981) 126(4):1562–8. doi: 10.1038/nri.2017.106
131. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of Early Viral and Bacterial Distribution and Disease by Natural Antibodies. *Science (80-)* (1999) 286(5447):2156–9. doi: 10.1126/science.286.5447.2156
132. Zhivaki D, Lemoine S, Lim A, Morva A, Vidalain P-O, Schandene L, et al. Respiratory Syncytial Virus Infects Regulatory B Cells in Human Neonates via Chemokine Receptor CX3CR1 and Promotes Lung Disease Severity. *Immunity* (2017) 46(2):301–14. doi: 10.1016/j.immuni.2017.01.010
133. Weber GF, Chousterman BG, Hilgendorf I, Robbins CS, Theurl I, Gerhardt LMS, et al. Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. *J Exp Med* (2014) 211(6):1243–56. doi: 10.1084/jem.20131471
134. Yates JL, Racine R, McBride KM, Winslow GM. T Cell–Dependent IgM Memory B Cells Generated during Bacterial Infection Are Required for IgG Responses to Antigen Challenge. *J Immunol* (2013) 191(3):1240–9. doi: 10.4049/jimmunol.1300062
135. del Barrio L, Sahoo M, Lantier L, Reynolds JM, Ceballos-Olvera I, Re F. Production of Anti-LPS IgM by B1a B Cells Depends on IL-1β and Is Protective against Lung Infection with Francisella tularensis LVS. *PloS Pathog* (2015) 11(3):e1004706. doi: 10.1371/journal.ppat.1004706
136. McKay JT, Haro MA, Daly CA, Yammani RD, Pang B, Swords WE, et al. PD-L2 Regulates B-1 Cell Antibody Production against Phosphorylcholine through an IL-5–Dependent Mechanism. *J Immunol* (2017) 199(6):2020–9. doi: 10.4049/jimmunol.1700555
137. Werwitzke S, Trick D, Kamino K, Matthias T, Kniesch K, Schlegelberger B, et al. Inhibition of lupus disease by anti–double-stranded DNA antibodies of the IgM isotype in the (NZB × NZW)F1 mouse. *Arthritis Rheumatol* (2005) 52(11):3629–38. doi: 10.1002/art.21379
138. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci* (2000) 97(3):1184–9. doi: 10.1073/pnas.97.3.1184
139. Díaz-Zaragoza M, Hernández-Ávila R, Viedma–Rodríguez R, Arenas-Aranda D, Ostoa-Saloma P. Natural and adaptive IgM antibodies in the recognition of tumor-associated antigens of breast cancer (Review). *Oncol Rep* (2015) 34(3):1106–14. doi: 10.3892/or.2015.4095
140. von Mensdorff-Pouilly S, Verstraeten AA, Kenemans P, Snijdewint FGM, Kok A, Van Kamp GJ, et al. Survival in Early Breast Cancer Patients Is Favorably Influenced by a Natural Humoral Immune Response to Polymorphic Epithelial Mucin. *J Clin Oncol* (2000) 18(3):574. doi: 10.1200/JCO.2000.18.3.574
141. Rauschert N, Brändlein S, Holzinger E, Hensel F, Müller-Hermelink H-K, Vollmers HP. A new tumor-specific variant of GRP78 as target for antibody-based therapy. *Lab Investig* (2008) 88(4):375–86. doi: 10.1038/labinvest.2008.2
142. Brändlein S, Eck M, Ströbel P, Wozniak E, Müller-Hermelink HK, Hensel F, et al. PAM-1, a natural human IgM antibody as new tool for detection of breast and prostate precursors. *Hum Antibodies* (2004) 13(4):97–104. doi: 10.3233/HAB-2004-13401
143. Goldstein MF, Goldstein AL, Dunsky EH, Dvorin DJ, Belecanech GA, Shamir K. Selective IgM immunodeficiency: retrospective analysis of 36 adult patients with review of the literature. *Ann Allergy Asthma Immunol* (2006) 97(6):717–30. doi: 10.1016/S1081-1206(10)60962-3
144. Yel L, Ramanuja S, Gupta S. Clinical and Immunological Features in IgM Deficiency. *Int Arch Allergy Immunol* (2009) 150(3):291–8. doi: 10.1159/000222682
145. Komai-Koma M, Gilchrist DS, McKenzie ANJ, Goodyear CS, Xu D, Liew FY. IL-33 Activates B1 Cells and Exacerbates Contact Sensitivity. *J Immunol* (2011) 186(4):2584–91. doi: 10.4049/jimmunol.1002103
146. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and Inflammation. *Cell* (2014) 157(1):121–41. doi: 10.1016/j.cell.2014.03.011
147. Macpherson AJ, McCoy KD, Johansen F-E, Brandtzaeg P. The immune geography of IgA induction and function. *Mucosal Immunol* (2008) 1(1):11–22. doi: 10.1038/mi.2007.6
148. Nakajima A, Vogelzang A, Maruya M, Miyajima M, Murata M, Son A, et al. IgA regulates the composition and metabolic function of gut microbiota by promoting symbiosis between bacteria. *J Exp Med* (2018) 215(8):2019–34. doi: 10.1084/jem.20180427

149. Lu LL, Suscovich TJ, Fortune SM, Alter G. Beyond binding: antibody effector functions in infectious diseases. *Nat Rev Immunol* (2018) 18(1):46–61. doi: 10.1038/nri.2017.106
150. Kubinak JL, Round JL. Do antibodies select a healthy microbiota? *Nat Rev Immunol* (2016) 16(12):767–74. doi: 10.1038/nri.2016.114
151. Longet S, Vonarburg C, Lötscher M, Miescher S, Zuercher A, Corthésy B. Reconstituted Human Polyclonal Plasma-derived Secretory-like IgM and IgA Maintain the Barrier Function of Epithelial Cells Infected with an Enteropathogen. *J Biol Chem* (2014) 289(31):21617–26. doi: 10.1074/jbc.M114.549139
152. Bioley G, Monnerat J, Lötscher M, Vonarburg C, Zuercher A, Corthésy B. Plasma-Derived Polyreactive Secretory-Like IgA and IgM Opsonizing Salmonella enterica Typhimurium Reduces Invasion and Gut Tissue Inflammation through Agglutination. *Front Immunol* (2017) 8:1043. doi: 10.3389/fimmu.2017.01043
153. Shen X, Miao J, Wan Q, Wang S, Li M, Pu F, et al. Possible correlation between gut microbiota and immunity among healthy middle-aged and elderly people in southwest China. *Gut Pathog* (2018) 10:4. doi: 10.1186/s13099-018-0231-3
154. Taborda CP, Casadevall A. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) Are Involved in Complement-Independent Antibody-Mediated Phagocytosis of Cryptococcus neoformans. *Immunity* (2002) 16(6):791–802. doi: 10.1016/S1074-7613(02)00328-X
155. Xander P, Vigna AF, Feitosa L dos S, Pugliese L, Bailão AM, et al. A surface 75-kDa protein with acid phosphatase activity recognized by monoclonal antibodies that inhibit Paracoccidioides brasiliensis growth. *Microbes Infect* (2007) 9(12):1484–92. doi: 10.1016/j.micinf.2007.08.001
156. Dufaud C, Rivera J, Rohatgi S, Pirofski L. Naïve B cells reduce fungal dissemination in Cryptococcus neoformans infected Rag1–/– mice. *Virulence* (2018) 9(1):173–84. doi: 10.1080/21505594.2017.1370529
157. Subramaniam KS, Datta K, Marks MS, Pirofski L. Improved Survival of Mice Deficient in Secretory Immunoglobulin M following Systemic Infection with Cryptococcus neoformans. *Infect Immun* (2010) 78(1):441–52. doi: 10.1128/IAI.00506-09
158. Elsegeiny W, Eddens T, Chen K, Kolls JK. Anti-CD20 antibody therapy and susceptibility to Pneumocystis pneumonia. *Infect Immun* (2015) 83(5):2043–52. doi: 10.1128/IAI.03099-14
159. Hirbod-Mobarakeh A, Aghamohammadi A, Rezaei N. Immunoglobulin class switch recombination deficiency type 1 or CD40 ligand deficiency: from bedside to bench and back again. *Expert Rev Clin Immunol* (2014) 10 (1):91–105. doi: 10.1586/1744666X.2014.864554
160. Milledge J, Kakakios A, Gillis J, Fitzgerald DA. Pneumocystis carinii pneumonia as a presenting feature of X-linked hyper-IgM syndrome. *J Paediatr Child Health* (2003) 39(9):704–6. doi: 10.1046/j.1440-1754.2003.00274.x
161. Sacks DL, Esser KM, Sher A. Immunization of mice against African trypanosomiasis using anti-idiotypic antibodies. *J Exp Med* (1982) 155 (4):1108–19. doi: 10.1084/jem.155.4.1108
162. Verdi J, Zipkin R, Hillman E, Gertsch RA, Pangburn SJ, Thomson R, et al. Inducible Germline IgMs Bridge Trypanosome Lytic Factor Assembly and Parasite Recognition. *Cell Host Microbe* (2020) 28(1):79–88. doi: 10.1016/j.chom.2020.04.012
163. Lun Z-R, Desser SS. Is the broad range of hosts and geographical distribution of Trypanosoma evansi attributable to the loss of maxicircle kinetoplast DNA? *Parasitol Today* (1995) 11(4):131–3. doi: 10.1016/0169-4758(95)80129-4
164. Krishnamurty AT, Thouvenel CD, Portugal S, Keitany GJ, Kim KS, Holder A, et al. Somatically Hypermutated Plasmodium-Specific IgM+ Memory B Cells Are Rapid, Plastic, Early Responders upon Malaria Rechallenge. *Immunity* (2016) 45(2):402–14. doi: 10.1016/j.immuni.2016.06.014
165. Lam JH, Baumgarth N. The Multifaceted B Cell Response to Influenza Virus. *J Immunol* (2019) 202(2):351–9. doi: 10.4049/jimmunol.1801208

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REVIEW
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B Cells in Patients With Melanoma: Implications for Treatment With Checkpoint Inhibitor Antibodies

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The contributions of the humoral immune response to melanoma are now widely recognized, with reports of positive prognostic value ascribed to tumor-infiltrating B cells (TIL-B) and increasing evidence of B cells as key predictors of patient response to treatment. There are disparate views as to the pro- and anti-tumor roles of B cells. B cells appear to play an integral role in forming tumor-associated tertiary lymphoid structures (TLSs) which can further modulate T cell activation. Expressed antibodies may distinctly influence tumor regulation in the tumor microenvironment, with some isotypes associated with strong anti-tumor immune response and others with progressive disease. Recently, B cells have been evaluated in the context of cancer immunotherapy. Checkpoint inhibitors (CPIs), targeting T cell effector functions, have revolutionized the management of melanoma for many patients; however, there remains a need to accurately predict treatment responders. Increasing evidence suggests that B cells may not be simple bystanders to CPI immunotherapy. Mature and differentiated B cell phenotypes are key positive correlates of CPI response. Recent evidence also points to an enrichment in activatory B cell phenotypes, and the contribution of B cells to TLS formation may facilitate induction of T cell phenotypes required for response to CPI. Contrastingly, specific B cell subsets often correlate with immune-related adverse events (irAEs) in CPI. With increased appreciation of the multifaceted role of B cell immunity, novel therapeutic strategies and biomarkers can be explored and translated into the clinic to optimize CPI immunotherapy in melanoma.

Keywords: melanoma, B cell, checkpoint inhibition therapy, antibody, humoral immune response

INTRODUCTION

During early stages, primary melanoma lesions are removable through surgical intervention that is largely curative. In advanced disease however, melanoma can spread to regional lymph nodes and metastasize to distant sites. Historically, treatment options were limited in advanced disease to palliative cytotoxic chemotherapy, leading to poor 5-year survival rates. Over the last decade, immunotherapy, driven by checkpoint inhibitor antibodies, has transformed patient prognosis.

The rationale behind the use of checkpoint inhibitor therapy lies in the highly immunogenic nature of melanoma (1, 2). Melanoma carries a large mutational load, providing a range of tumor-specific antigens that are thought to elicit a host immune response. However, melanoma cells can evade immunosurveillance *via* activation of different immune-inhibitory pathways, including *via* immune checkpoint molecules and their downstream signals. Physiologically, checkpoint pathways play a role in immune homeostasis, providing negative feedback stimuli to prevent autoimmune reactivity. The best-studied checkpoint pathways are those of negative regulatory molecules cytotoxic T lymphocyte associated protein-4 (CTLA-4) and of the Programmed Death Receptor 1 (PD-1) and its ligands PD-L1 and PD-L2.

Checkpoint inhibitors (CPIs) were designed to promote immune-mediated elimination of tumor cells through modulation of T cell responses. Anti-CLTA-4 (Ipilimumab approved in 2011) (3) or anti-PD-1 (Pembrolizumab and Nivolumab approved in 2014) (3, 4) antibodies inhibit binding of checkpoint molecules with their respective ligand in the tumor microenvironment or draining lymph nodes. A newer anti-PD-L1 agent, Atezolizumab, was approved by the FDA in July 2020 (5). CPIs are approved for use as monotherapy in metastatic melanoma and as adjuvant therapy. More recently combination Nivolumab and Ipilimumab has been approved for metastatic disease in select patient groups. Their clinical efficacy and long-term outcomes are highlighted in the CheckMate 067 clinical trial (ClinicalTrials.gov NCT01844505) (4). In 945 patients with stage III or IV melanoma, this trial directly evaluated combination therapy with Nivolumab plus Ipilimumab in comparison with monotherapy with Nivolumab or with Ipilimumab. At a minimum follow-up of 60 months, the median overall survival (OS) was more than 60.0 months (median not reached) in the Nivolumab-plus-Ipilimumab group, 36.9 months in the Nivolumab group and 19.9 months in the Ipilimumab group. The 5-year OS rates were 52, 44, and 26% respectively (4).

Despite the undeniable clinical success of CPI therapy several challenges remain. There is currently a lack of biomarkers, limiting our ability to predict who will respond to treatment. Another important consideration is recognizing who will develop toxicities that are unfortunately common particularly with anti-CTLA-4 treatment and in the context of combination anti-CTLA-4 and anti-PD-1 therapy as compared with monotherapy. These toxicities, known as immune related adverse events (irAEs) vary in severity, can affect any organ system but most commonly target the skin, the gastrointestinal and the endocrine systems (6). Although many of these toxicities are manageable/reversible, they frequently lead to treatment disruption or discontinuation. Therefore, understanding the mechanisms underlying irAEs and predicting these irAEs is a much-needed clinical strategy.

The role of T cells in CPI therapy has been extensively reviewed in literature. Contrastingly, B cell immunity has been less studied. B cells have a wide range of roles, including critical functions as professional antigen presenting cells (APCs), and they are capable of secreting cytokines and antibodies which enable them to conduct antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). Considering their comprehensive functions and with B cells making up one of the two arms of the adaptive immune system, it is not surprising to envisage a role within melanoma immunity and response to CPI. However, the exact role B cells play in tumor immunity is unclear with the existence of different cell subsets with juxtaposing functions, such as activatory and regulatory B cells (Bregs), and the presence of B cells in tertiary lymphoid structures (TLSs). The potential pro- and anti-tumor roles of B cells in melanoma are summarized in **Figure 1**. In this review we discuss emerging evidence for the significance of B cell and antibody immune responses in melanoma and specifically in the context of CPI therapy.

THE CTLA-4 AND PD-1 PATHWAYS

CTLA-4 is an inhibitor of T cell activation primarily expressed on naïve T cells after activation (7–10) and constitutively expressed on FoxP3+ regulatory T cells (Tregs) (10). T cell activation is dependent not only on T cell receptor (TCR) binding with an antigen presented *via* an APC, but also on the presence of a costimulatory second signal, typically through binding of CD28 on the T cell to CD80/86 on the APC. CTLA-4 is a competitive CD28 homolog that has a higher affinity to CD80 (B7-1) and to a lesser degree CD86 (B7-2) than CD28 therefore inhibiting T cell co-stimulation (10). TCR signaling up-regulates cell surface CTLA-4 expression, reaching peak expression at 2 to 3 days after activation (7, 8). This provides a negative feedback loop for T cell activation.

PD-1 is also a member of the immunoglobulin superfamily known to contribute to immune homeostatic processes by delivering inhibitory signals upon engagement with its ligands, programmed death ligands 1 and 2 (PD-L1/2). Like CTLA-4, PD-1 is thought to be a negative regulator of T cell function, regulating peripheral tolerance and T cell responses. PD-1 is expressed more broadly than CTLA-4 and can be found on T cells, B cells, natural killer (NK) cells, and a variety of peripheral tissues (11). Expression of PD-1 is up-regulated upon activation of T cells and B cells (12). The ligand PD-L1 is broadly expressed by immune cells including T cells, B cells, dendritic cells and macrophages, and in non-lymphoid tissues including on tumor cells or stromal elements in the tumor microenvironment (TME) (11, 13). Recent evidence suggests that CD28 is also a primary target for PD-1 (14, 15).

Intracellularly, both PD-1 and CTLA-4 signal *via* SHP2 and converge to inhibit downstream P13K signaling (16, 17).

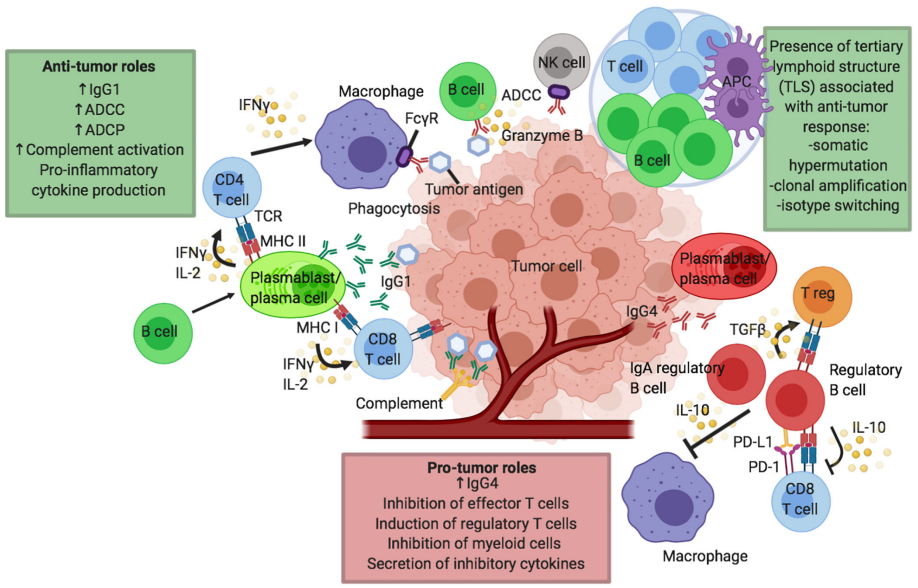

FIGURE 1 | Proposed humoral responses in cancer and in response to checkpoint inhibitor immunotherapy. Putative pro- and anti-tumor roles of B cells in the tumor microenvironment (TME) and tumor tertiary lymphoid structures (TLS). B cells may confer several anti-tumor roles through production of effective tumor-clearing antibodies, predominantly IgG1: mediating antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP) and facilitating complement activation. B cells can also effectively modulate CD4 and CD8 T cells *via* their functions as antigen presenting cells (APCs) to present tumor-derived antigens or by promoting cytokine secretion, particularly IFNγ and IL-2 by T cells. B cells can also support the presentation of tumor-derived antigens by other antigen presenting cells including macrophages and dendritic cells. Antigen presentation and B cell activation may be enhanced in TLS, leading to somatic hypermutation, clonal amplification and isotype switching to activatory antibody isotypes such as IgG1, further driving anti-tumor responses. B cells can also directly exert cytotoxic functions against tumor cells (*e.g.* through secretion of granzyme B). B cells can have pro-tumor roles by multiple mechanisms, for instance through expression of inflammatory antibody isotypes such as IgG4. IL-10 producing regulatory B cell (Breg) phenotype is described which can negatively modulate the function of effector CD8 T cells, induce the development of regulatory T cells (Tregs) and dampen the activity of antigen-presenting cells such as dendritic cells and macrophages. IgA producing regulatory B cells has also been described in the TME.

Although it is reported that B cells express PD-1 and PD-L1, these cells have not been recognized as a primary target for anti-PD-1 therapy. CTLA-4 also acts extracellularly to clear the CD28 ligands CD80/86 from surrounding cells, including APCs, by trans-endocytosis *in vivo*, further inhibiting T-cell activation (18). While B cells are also known to express CTLA-4 ligands CD80/CD86, again they are not considered the key target cells of anti-CTLA-4 therapy (10).

OVERVIEW OF B CELLS IN MELANOMA, IN THE TUMOR MICROENVIRONMENT, AND TERTIARY LYMPHOID STRUCTURES

Besides constituting an amalgamation of malignant cells, the tumor microenvironment (TME) can be thought of as a distinct complex "organ" which contains an array of different cells, such as immune cells, tumor-associated vasculature and lymphatics, fibroblasts and pericytes (15). The immune cell infiltrates are of special importance as they often correlate with anti-tumor activity and have been reported to be associated with a more favourable prognosis in some settings. Melanoma is a classically highly immunogenic tumor (19) and lymphocyte infiltrates in general have been shown to have positive prognostic value in patients with primary cutaneous melanoma (20). Immune cell subsets which are correlated with increased patient survival outcome include CD8+ memory T cells, CD4+ T-helper cells (21), B cells (22) and NK cells (23). Immune cell infiltrates harbor the potential to exert cytotoxic anti-tumor activity, which may be exacerbated by checkpoint inhibitor immunotherapy.

Lymphocyte activation typically occurs in secondary lymphoid structures which include lymph nodes, the spleen and mucosal-associated lymphoid tissue (24). During chronic inflammation, TLS may transiently form, which confer sites enriched in B and T lymphocytes and dendritic cells present from disordered compositions to more structured and often highly specialized germinal center-like formations (25). As tumors share many of the characteristics of chronic inflammation, it is unsurprising that TLSs have been identified in a range of cancers adjacent to the tumor site, including in 26% of metastatic melanomas (26). Importantly, the presence of TLS has been shown to be associated with a more favorable prognosis (27, 28) and the promotion of immunotherapy response (discussed below) (29). There is compelling evidence of ongoing B cell maturation in these sites, driving clonal amplification, class switching and somatic hypermutation (SMH). It is therefore clear that intralesional B lymphocyte structural organization and the interaction with other

lymphocytic cell subsets within TLS could be of particular importance to overall B cell-mediated anti-tumor activity.

The majority of melanoma lesions contain significant populations of B cells, usually localized within the tumor stroma (one study has reported that 86% of primary melanomas harbor at least ten $CD20^+$ stromal B cells per mm^2 tumor (30). Furthermore, tumor infiltrating B lymphocytes TIL-B in primary lesions make up an average of 10% of the total infiltrating lymphocytes (31–33) and can be found adjacent to various other immune cell subsets. $CD22^+$ B cells are enriched in melanoma lesions compared to healthy skin, and CD20 and CD22 mRNA expression is further enhanced with metastasis (34, 35), indicating the presence of a pronounced humoral immune response in melanoma.

These TIL-Bs have been shown to contribute to anti-tumor immunity *via* antibody responses to melanoma-associated antigens. In one study, 28% of melanoma patient-derived B cell cultures were capable of binding to a wide range of antigens expressed by melanoma cell lines, as opposed to just 2% of those derived from healthy individuals, and this was shown to further increase with metastatic disease (36). In concordance, plasma cells in melanoma lesions were shown to be polyclonal and to predominantly express IgG and IgA (37) implicating antigen reactivity and class switching, and thus a possible melanoma-reactive B cell immune response. Secreted immunoglobulins, especially of the IgG1 subclass, may be able to exert potent tumoricidal effects *via* ADCC, as demonstrated *in vitro* using a patient-derived monoclonal antibody (36). ADCC however is just one of several effector mechanisms mediated by secreted immunoglobulin. A neutralizing anti-human growth factor (HGF) antibody has been shown to suppress tumor growth *in vitro* (38). B cells may also act as antigen presenting cells, contributing to T-helper cell activation and anti-tumor effector mechanisms (39). Finally, activatory B cells may facilitate anti-tumor immunity *via* the release of pro-inflammatory cytokines such as IL-2, TNFα and IFNγ (40, 41). TNFα produced by B cells may control the development of follicular dendritic cells, the formation of B cell follicles and T cell-dependent antibody responses (42). Collectively, several studies therefore support the presence of an antigen-specific B cell immune response to melanoma.

In accordance with expectations arising from these anti-tumor properties, the percentage of tumor-infiltrating and peritumoral B cells have been found to positively correlate with more favorable patient survival in the vast majority of melanoma cohort studies (22, 43, 44). However, some studies have in contrast found no significant association between B cell infiltration and overall patient survival (31), and even a correlation of B cell infiltrates with a poorer prognosis (33). These discrepancies may arise from differences in experimental design and analyses (*e.g.* using raw B cell counts *versus* percentage of infiltrating populations) or may reflect the confounding presence of immunosuppressive regulatory B cells.

B CELL SUBSETS IN MELANOMA AND THE ROLE OF BREGS

Several studies have demonstrated the enriched presence of various differentiated B cell phenotypes such as memory, plasmablasts (PBs) and plasma cells within melanoma tumors. Elevated percentages of PBs (short-lived cells and part of the rapid antigen response) have been reported in the circulation of patients with melanoma compared to blood from healthy individuals. PBs are short-lived cells and part of a rapid antigen response. Although PBs have a potentially positive role in melanoma, the functional plasticity of these cells may result in transient switching to a "regulatory" pro-tumor phenotype owing to expression of TGFβ and IL-10 by this cell subset (41).

IL-10-producing "regulatory" B (B10) cells (45) have been shown to be functional in murine models of tolerance (46), in preventing chronic inflammation (47–50) and in curbing anti-tumor immune responses (51). In mouse models, Bregs appear to be derived from either BII (follicular) or Marginal Zone (MZ) cells. A $CD1d^+$ $CD19^{++}$ regulatory B cell subset has been identified in mice which strongly resembles MZ cells and protects against colitis, through interactions with Tregs (52). These observations not only demonstrate that these cells play a crucial pathogenic role in health and disease, but also that B cells can contribute to immune outcomes in general *via* their polarized cytokine expression.

Studies in mouse models of skin cancer suggest that there are certain circumstances where B cells can exert a distinctly immunosuppressive pro-tumor effect (53). A subset of "tumor-evoked" Bregs has been shown to facilitate the metastasis of breast cancer tumors through the induction of Tregs *via* TGFβ secretion (54). Importantly, tumor-associated antigens such as 5-lipoxygenase (breast cancer (55); and placental growth factor (glioblastoma (56); have been shown to induce IL-10-production in tumor-infiltrating B cells in murine models. It may therefore be possible that B cells in the tumor-microenvironment are coerced into developing a regulatory phenotype ($TGF\beta^+$ and $IL\text{-}10^+$), and that tumor-derived antigens in general could induce B cell IL-10-expression (57). As expected, Bregs have been shown to directly contribute to tumor-progression as a result of their anti-inflammatory properties (58).

At present there is a lack of substantial information relating to the role of immunosuppressive IL-10-producing B cells in affecting tumor progression in humans. Perhaps the most significant study showed that Granzyme-B-expressing IL-10+ B cells induced by T cells IL-21 secretion infiltrate solid tumors of breast, cervical, and ovarian cancer patients and inhibit T cell responses (59). In addition, IL-10+ transitional B cells have been shown to be up-regulated in gastric cancer patient peripheral blood and tumors and are functionally capable of suppressing Th1 cytokine secretion by T cells and inducing Tregs (60).

The current evidence for regulatory B cells in melanoma tissue microenvironments rests upon a study demonstrating that adoptive transfer of B1a B cells (possessing an IL-10+ regulatory phenotype) into wild-type mice significantly exacerbated B16F10 melanoma growth, illustrating that IL-10-producing B cells can directly contribute to melanoma tumor-progression (61). A separate murine model of squamous cell carcinoma obtained similar conclusions (62). Finally, Bregs have been shown to supress cutaneous inflammation in a mouse model of psoriasis-like inflammation (63). To date, there have been no

investigations into the role of IL-10-producing B cells in patients with melanoma.

Tumor-infiltrating regulatory B cells therefore represent a potential mechanism of tumor-mediated immune escape that in certain conditions may outweigh antigen-presentation and anti-tumor antibody-mediated effector mechanisms to skew the overall impact of B cell infiltrates towards a neutral or even a negative effect, and mandates studies into the induction of regulatory B cells in melanoma patients and correlation with overall survival.

INFLUENCE OF THE TH2-BIASED MELANOMA MICROENVIRONMENT ON PRO-TUMOR PROPERTIES OF B CELLS

Consistent with the contributions of regulatory B cells, the microenvironments of melanoma solid tumors are characteristically considered as harboring Th2-biased cytokine expression profiles (64), typical of chronic inflammatory conditions. These microenvironments typically confer pro-tumor properties including promoting angiogenesis and inhibiting cell-mediated responses, such as those mediated by cytotoxic T-lymphocytes (CTLs). The classic Th2 cytokine is interleukin-4 which, in addition to interleukin-13, may induce tumor clearance (65). However, IL-10 produced by Bregs, Tregs and M2-type macrophages in the tumor microenvironment can, in addition to its potent suppressive effects upon CTLs, trigger a modified or alternative Th2 response by inducing B cell IgG4 subclass switching in the presence of IL-4 (66).

Indeed, it has been shown *in vitro* that these Th2-biased microenvironments favor alternatively activated humoral immunity which confers a shift in B cell antibody expression towards IgG4 (67). Early evidence has accordingly demonstrated that IgG4 serum levels are dysregulated in melanoma patients, particularly in advanced disease settings (68). IgG4 subclass antibodies are structurally distinct to IgG1 antibodies and confer a weaker ability to bind and stimulate effector cells such as macrophages to initiate ADCP and ADCC through their Fc-gamma receptors (69). It has been shown that these antibodies compete with IgG1 for Fc-gamma receptor sites resulting in a reduced degree of immune cell activation and tumor-cell killing when secreted in large quantities (34, 67). Recent evidence also suggests that Fc–Fc interactions between IgG4 and cancer-specific IgG1 antibodies may confer an important aspect of tumor immune evasion (70). Interestingly, the same study also demonstrated that the IgG4 subclass CPI antibody Nivolumab participated in Fc-interactions with IgG1 subclasses and promoted cancer growth in a murine model of colon cancer. The pro-tumor role of IgG4 subclasses may appropriately explain observations of possible hyperprogressive disease seen in some CPI patients.

IgG4 is therefore a negative prognostic indicator of patient overall survival, and these Th2 biased microenvironments offer a form of immune evasion which is mediated by IL-10 (71, 72). Interestingly, recent evidence has also revealed that an IgG4-expressing CD49b+ CD73+ B cell subset expressing proangiogenic cytokines including VEGF is up-regulated in the circulation of melanoma patients, highlighting an additional and previously unknown mechanism by which tumor-infiltrating B cells may contribute to tumor progression (73).

THE ROLE OF B CELLS IN CHECKPOINT INHIBITOR THERAPY RESPONSES

Checkpoint inhibitors are designed to regulate T cell effector functions; however, there is growing interest in the contribution of B cell responses to patient outcomes. This has been examined in several recent studies; the findings are summarized in **Table 1** and **Figure 2**.

Tumor-Infiltrating B Cell Signatures in Relation to CPI Treatment Response

Further evidence supports the notion that specific B cell subsets may hold prognostic value in CPI treatment response in advanced melanoma. B cell receptor repertoire and B cell phenotype were analyzed in 473 cutaneous melanoma specimens. The absence of B cell immunoglobulin heavy chain γ (IGHG) gene was found to correlate with lack of response to anti-CTLA-4 but this was not shown in the context of anti-PD-1 therapy (22). In addition, a higher proportion of IL-10 secreting B cells was associated with lack of response to anti-CTLA-4, but was not correlated with response to anti-PD-1 therapy (22). A phase 2 trial of neoadjuvant CPI [neoadjuvant Nivolumab (n = 11) *versus* neoadjuvant Nivolumab plus Ipilimumab (n = 12)] (78) also highlighted the importance of B cells in CPI response. While baseline tumor-infiltrating B cell counts did not differ significantly between responders and non-responders in this small sample size, early on-treatment tumor samples showed increased tumor-infiltrating B cell frequency (as defined by CD20 immunostaining) in treatment responders compared with non-responders. Helmink et al. used the same cohort and performed bulk RNA sequencing from baseline and early on-treatment tumor biopsy samples (29) and found that B cell markers were the most differentially expressed genes in responders to therapy compared with non-responders. They identified significantly increased clonal counts for both immunoglobulin heavy and light chains and increased BCR diversity in responders than in non-responders, which suggests an active role for B cells in anti-tumor immunity. Pathways up-regulated in responders as compared to non-responders included those consistent with increased immune activity such as CXCR4 signaling, cytokine receptor interaction and chemokine signaling pathways. Phenotyping of B cells in tumors identified significantly higher frequency of memory B cells in responders *versus* non-responders at baseline and early on-treatment. They corroborated their findings in a further cohort of 18 patients with melanoma treated with adjuvant or neoadjuvant CPI. Unlike tumor-derived B cells, a peripheral blood B cell phenotype was not found to correlate with treatment outcome (29). Since most samples in this study were blood or metastatic lymph node deposits, the cohort was relatively small and the samples

TABLE 1 | Summary of current evidence of a humoral immune response in patients treated with checkpoint inhibitor therapy.

Sample type	Time point	CPI used	Target	B cell and antibody responses	Reference
Tumor	Baseline	Ipilimumab	CTLA-4	■ IL10+ Breg enrichment found in non-responders to anti-CTLA-4 therapy ■ Absence of IGHG gene signature enriched in non-responders to anti-CTLA-4	(22)
		Nivolumab	PD-1	■ IL10+ Breg presence did not correlate with treatment response to anti-PD-1 ■ IGHG gene signature did not correlate with treatment response to anti-PD-1	
Tumor	Baseline	Ipilimumab & Nivolumab combination	CTLA-4 & PD-1	■ For all treatments increased B cell frequency found in the tumours of responders to both combination therapy and anti-PD-1 monotherapy in both baseline and early on treatment samples ■ For all treatments higher density of CD20+ B cells and TLS and a higher TLS:tumour area ratio in early on-treatment samples from responders versus non-responder ■ For all treatments increased frequency of switched memory B cells found in responders at baseline and early on treatment	(29)
		Nivolumab	PD-1		
	Early on treatment	Ipilimumab & Nivolumab combination	CTLA-4 & PD-1		
		Nivolumab	PD-1		
Tumor	Baseline	Ipilimumab	CTLA-4	■ B cells found to localise within TLS ■ TLS gene signature present in responders'' ■ TLS structures associated with CD8+ T cells	(27)
Peripheral blood	After 1 year of treatment	Ipilimumab	CTLA-4	■ High plasmablast numbers seen in patients responding to anti-CTLA-4 ■ Sequenced plasmablast antibodies in patients who had not progressed after 1 year had somatic hypermutation, class switching and clonal expansion showing an active humoral response	(74)
Tumor	Baseline	Nivolumab	PD-1	■ Plasmablast-like phenotype (CD19+CD20+CD38+CD138-) found to be most common B cell phenotype in TME ■ Tumour induced plasmablast population (TIBP) defined using melanoma secretome ■ Increased expression of TIPB gene signature in baseline samples associated with improved response to Nivolumab	(41)
Peripheral blood	Baseline	Nivolumab	PD-1	■ Melanoma-antigen specific antibodies to: MDA (TRP1/TYRP1, TRP2/TYRP2, gp100, MelanA/MART1) and the Cancer-Testis antigen NY-ESO-1 higher in responders to all treatments ■ Melanoma-antigen specific IgGs higher in responders at baseline compared with non-responders for all treatments ■ Melanoma-antigen specific IgGs in patients were mainly IgG1 and IgG2	(75)
		Pembrolizumab	PD-1		
		Ipilimumab	CTLA-4		
		Ipilimumab & Nivolumab combination	CTLA-4 & PD-1		
Peripheral blood	Baseline	Nivolumab	PD-1	■ For all treatments high titres of IgG, IgG1, IgG2 and IgG3 showed a positive correlation with progression-free survival (PFS) ■ For all treatments high titre of IgG2 showed a positive correlation with overall survival (OS)	(76)
		Pembrolizumab	PD-1		
		Ipilimumab	CTLA-4		
		Ipilimumab & Nivolumab combination	CTLA-4 & PD-1		
Peripheral blood	Baseline	Ipilimumab & Nivolumab combination therapy	CTLA-4 & PD-1	■ Overall decline in B cell numbers but increased plasmablast and $CD21^{lo}$ B cell subsets corresponded to development of high grade immune related adverse events (irAE) ■ Changes in other immune cells not significantly associated with frequency or severity of irAE	(77)
	Early on-treatment				

were sourced either at baseline or early on-treatment; these findings would benefit from evaluations of larger and more comprehensive studies.

Furthermore, in a cohort of 39 patients with advanced melanoma, early changes were observed in peripheral B cell subsets upon one cycle of combination CPI therapy (Ipilimumab and Nivolumab): particularly a significant decrease in circulating B cells in patients who underwent combination CPI as opposed to those treated with a monotherapy anti-CLTA-4 or anti-PD-1 (77). No significant differences were found between naïve and memory B cell sub-populations. Despite the overall decline in B cell numbers, a marked increase was observed in the density of: class-switched memory B cells, plasmablasts and $CD21^{lo}$ B cell sub-populations (77). Of note, significant changes in the latter

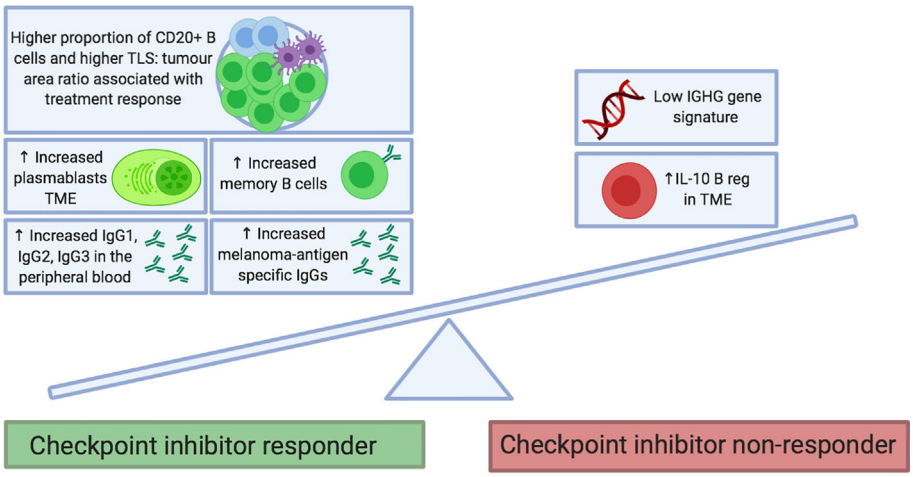

FIGURE 2 | Proposed B cell features at baseline that predict response to checkpoint inhibitor treatment. B cell phenotypes enriched in tumors and the circulation of patients before treatment may be predictive of clinical response to checkpoint inhibitor therapy. Features include the presence of memory B cells (*e.g.* Ipilimumab monotherapy and combination of Ipilimumab and Nivolumab) and plasmablasts (*e.g.* Ipilimumab and Nivolumab monotherapies). In the peripheral blood, immunoglobulin isotypes IgG1, IgG2 and IgG3 have been associated with response to Ipilimumab and Nivolumab monotherapies. Melanoma-antigen specific IgG antibodies have been found to be increased in responders to therapy (monotherapy with Ipilimumab, Pembrolizumab and Nivolumab; as well as with combination of Ipilimumab and Nivolumab). Conversely, enrichment of tumors with IL10+ regulatory B cells and an absence of an IGHG gene signature have been associated with a poor response to Ipilimumab. FcγR, Fcγ receptor; NK, natural killer; TCR, T cell receptor.

were only exhibited in patients who underwent combination or anti-CTLA-4 treatment. This is consistent with findings from a previous study whereby loss of circulating B cells and increase in $CD21^{lo}$ B cells were reported in patients treated with an anti-CTLA-4 agent (79). Detailed evaluation of the $CD21^{lo}$ B cell compartment revealed equal concentrations of naïve and memory B cells at baseline, but moderate increase in memory B cells after combination checkpoint inhibition. Furthermore, B cell receptor sequencing demonstrated that $CD21^{lo}$ B cells possessed higher: clonality, maximal clone frequency, and SHM frequency. Therefore, $CD21^{lo}$ B cells were shown as a phenotypically distinct B cell subset present in melanoma patients, which increased in frequency following combination checkpoint inhibition and suggest an active B cell response in the TME. PD-1 expression on the $CD21^{lo}$ B cells was higher than on other B cell subsets, suggesting that this population may be specifically modulated by anti-PD-1 therapy. This study failed to find an association between B cell changes and clinical response. The extrapolation of these findings may be limited due to the small sample size and the fact that on-treatment peripheral blood samples were only taken at an early time point after commencement of CPI therefore may not be representative of the full response.

CPI-Associated Modulation of Plasmablasts

There is some evidence that plasmablasts (PB) may be directly modulated by CPI. Circulating plasmablast levels were significantly enhanced in patients undergoing treatment with anti-CTLA-4 immunotherapy (74). When immunoglobulins produced by the PBs were sequenced, there was evidence of clonal expansion, SMH and class switching, indicative of an active B cell response (74). Immunostaining of 41 metastatic melanoma specimens revealed that a plasmablast-like phenotype (CD19+CD20+CD38+CD138−) is the most frequently observed B cell subset in the TME (41). Furthermore, circulating B cells could be induced to differentiate into plasmablast-like cells when exposed to autologous melanoma secretomes *in vitro* (41). In this study tumor-associated B-cells also expressed T-cell and macrophage chemoattractant mediators (CCL3, CCL3L1, CCL4, CCL5, CCL28, and CXCL16) suggesting that B-cells can orchestrate the immune response in the TME by modulating other cell populations. Increased expression of immunosuppressive cytokines IL-10 and TGFβ1 was reported in plasmablast-like and memory B cell sub-populations, consistent with a potential regulatory role. However, these signatures may also be associated with treatment response: proteomics and RNA-seq data were used to define a tumor-induced plasmablast-like population (TIBP) functional signature, and this signature was evaluated in a historical dataset from patients treated with Nivolumab. In this analysis, the presence of this TIBP signature at baseline correlated with better response to anti-PD-1 treatment (41).

A Role for Tertiary Lymphoid Structures

The importance of TLS in response to CPI has also been investigated. Helmink et al. delved deeper into the localization of B cells within their original cohort of 23 tumors and identified a higher density of CD20+ B cells in TLS and a higher TLS:tumor area ratio in early on-treatment samples from responders *versus* biopsies from non-responders (29). They also illustrated that CD20 B cells often co-localized with CD4, CD8 and FOXP3 T cells. A second study also identified CD20 B cells in 25% of 177

melanoma specimens (27). CD20 B cell clusters were surrounded by CD4 T cells. Increased expression of known molecular markers of TLS (CXCL13, CXCR5 and DC-LAMP) was described and immunostaining confirmed the co-localization of CXCL13 and CXCR5 with the CD20 B cell marker. A TLS gene signature in melanoma was defined and integrated into four historical melanoma cohorts treated with CPI. This TLS gene signature at baseline was associated with significantly increased survival with anti-CTLA-4 therapy, and this was independent of the tumor mutational burden which may be a predictor for TLS formation (27). The role of B cells in TLS in response to CPI has also been highlighted in a third study in the treatment of sarcoma (80). In this, 213 soft tissue sarcomas were stratified according to the composition of the TME. Those with highest density of B cells and TLSs were associated with the highest response rate to CPI. The study identified several immune cell-related gene signatures and the only one significantly associated with extended overall survival was the B cell signature (80).

Taken together these studies highlight that B cell phenotype, localization with TLS and B cell gene expression may be directly modulated by CPI and could be relevant to treatment outcomes. The differential results seen with different agents and combination therapy likely reflect the distinct mechanisms of action of each treatment (81).

ANTIBODY RESPONSES IN MELANOMA

A key feature of B cells is their ability to produce antibodies. B cells in the tumor can undergo class switching and affinity maturation, producing tumor-specific or non-specific antibodies of different isotypes (24) and could exert anti-tumor or pro-tumor roles (82). Human antibodies are tetrameric glycoproteins formed by two heavy and two light polypeptide chains. Heavy and light chains assemble in a Y shaped structure, defined by a Fab region (formed by two identical Fab fragments, composed by the light chain and part of the heavy chain), an Fc region (formed by the constant portion of the two heavy chains) and a hinge region (joining Fab and Fc regions). The variable regions of heavy and light chains are assembled to form two identical antigen binding sites. The heavy chain constant region determines the isotype of the antibody, with a characteristic hinge and Fc region. The hinge region is responsible for the flexibility of the Fab arms which can affect both antigen binding and effector function and for some isotypes, such as IgG4, these can be involved in a process called Fab Arm Exchange, which results in bispecific (IgG4) antibodies (72). The Fc region is involved in the binding to Fc receptors (FcRs) and C1q (complement) and is responsible for antibody mediated effector functions such as ADCC, ADCP and CDC (81).

Depending on their isotype and specificity, antibodies can have opposite roles in the tumor-associated immune response. Different antibody isotypes have different affinity for FcRs and C1q and consequently have different abilities to trigger effector functions. Tumor-specific antibodies with high affinity for FcRs and C1q (such as IgG1) have the potential to engender anti-tumor effects, mediating ADCC, ADCP, CDC or to support antigen presentation by mediating the uptake of tumor antigens by APCs such as macrophages and dendritic cells (82). Furthermore, membrane bound tumor-specific antibodies on the surface of B cells can engage with tumor cells and trigger B cell mediated cytotoxicity. Evidence of B cell mediated cytotoxicity *via* granzyme B and TRAIL has been shown in hepatocellular carcinoma (83). IgG class antibodies have been found to be tumor-specific, and this has been reported in melanoma (75) and other cancer types and has been correlated with a favorable prognosis (84–87).

Tumor-associated antibodies can also have either pro-tumor effects or their anti-tumor effects may be regulated by tumors. Tumor-specific antibody-mediated immune cell activation and anti-tumor cytotoxicity may also be impaired by expression of inhibitory Fc receptors (FcγRIIbs) in the TME and downstream immunoreceptor tyrosine-based inhibitory motif (ITIM) domain signalling (88, 89). Tumor-specific antibodies may be expressed as isotypes (such as IgG2 or IgG4) with poor affinity for activating FcRs or complement. These isotypes can be ineffective in triggering anti-tumor responses like ADCC, ADCP, CDC and antigen presentation (72). Furthermore, IgG4 antibodies can engage the inhibitory receptor FcγRIIb. This might further impair anti-tumor responses, blocking IgG1 mediated effector function (67). Tumor-associated antibodies not specific for the tumor can also serve as a decoy blocking other potentially immuno-activating and tumor-reactive antibodies from engaging with immune effector cells. This can contribute to pro-tumor effects such as ineffective T cell mediated responses or B effector functions. It is also possible that tumor-specific antibodies accumulating at the tumor site over time forming immune-complexes, promoting chronic inflammation and immunosuppressive myeloid cell phenotypes (82).

Isotypes like IgG1 and IgG3 have high capacity to trigger ADCC and facilitate complement activation. The IgG1 isotype is usually associated with anti-tumor responses while TCGA RNA-seq data analysis of melanoma samples showed that IgG3 is either neutral or is associated with a negative prognosis. This could be explained by the fact that IgG3 has a shorter half-life (1 week) compared to other isotypes (IgG1, 2 weeks). Furthermore B cells expressing IgG3 isotype antibodies usually undergo less antibody SHM, and therefore IgG3 antibodies may have lower affinity for the target antigen while still able to occupy FcRs (90). IgG2 has poor ability to bind FcRs and C1q. Evidence of antibody responses switched to IgG2 has been found in melanoma (26). IgG4 has poor ability to bind activating FcRs while able to bind the inhibitory receptor FcγRIIb and has complement-activating ability. Evidence of IgG4 antibody responses has been found in the melanoma TME, and serum IgG4 has been associated with poor prognosis in melanoma (36, 67, 71).

TCGA RNA-seq data analyses also suggest that IgD, IgE, and IgA antibodies may be associated with poor prognosis in melanoma (90). One possible explanation is that IgD can bind basophils resulting in the production of cytokines like IL-4, IL-5,

IL-13, BAFF, and APRIL which may support class switching to IgA and IgE and a Th2 immune response. Furthermore, the presence of IgE antibodies is usually associated with IL-4, which can further support a Th2 immune response. IgA antibodies have been found in melanoma and are associated with poor prognosis. This could be explained by the immune-suppressive phenotype of IgA+ B cells, expressing IL-10 and PD-L1 and by the ability of IgA+ B cells to support the expansion of T regulatory (Treg) cells which in turn secrete TGFβ supporting IgA class switch. Melanoma-associated IgA has been found to correlate with poor clonality, suggesting that, in the tumor, class switching to IgA is a consequence of inflammation and not of an antigen-driven response, and that tumor-associated IgA could be non-tumor specific (82).

ASSOCIATIONS OF B CELL EXPRESSED ANTIBODIES WITH CPI TREATMENT

Based on mounting evidence in support of potential roles of various antibody isotypes in melanoma, it is not surprising that antibody responses have been studied and are thought to correlate with outcome of CPI therapy. In the context of B cells in HIV infection, blocking of PD-1 has been shown to increase viral antigen-specific antibody responses (91). This may lend merit to the notion that PD-1-specific CPI could improve tumor-specific antibody responses (92).

One study investigated whether IgG antibody and isotype levels correlated with anti-tumor response and survival following CPI therapy. IgG subclass analysis was performed on serum samples at baseline from 49 patients with melanoma treated with anti-PD1 antibody (Nivolumab or Pembrolizumab (86%), Nivolumab plus Ipilimumab (10%), or Ipilimumab (4%). A positive correlation with progression-free survival (PFS) was found for high titers of total IgG, IgG1, IgG2, and IgG3, while a positive correlation with overall survival (OS) was found to be significant only for the IgG2 subclass (76). This result is quite interesting since IgG2 is usually not associated with anti-tumor immune responses due to its poor ability to trigger ADCC, ADCP, CDC and facilitate antigen presentation, but may reflect activation of a tumor antigen-specific response triggered by CPI.

A different preliminary study compared the antibody sequence repertoires of 26 patients with melanoma treated with Pembrolizumab (9), Ipilimumab (8) or Nivolumab, and Ipilimumab combination (9). It was found that long-term responders had higher levels of SHM after initiation of treatment, and on-treatment compared to the non-responders. Comparison of lineages between patients identified antibodies with high sequence similarity suggesting these antibodies may have arisen from convergent selection, *i.e.*, different patients raising antibodies against shared or similar epitopes. Moreover, when antibody lineages were analyzed and compared among patients, antibodies with high sequence similarity were found, suggesting these antibodies may have arisen from convergent selection and that the patients may be producing antibodies against shared epitopes. Of note, IgG2 was the most frequent isotype of these antibodies. Furthermore IgG2 was higher in responders compared to non-responders (93).

In a different study, melanoma patients treated with CPI (monotherapy with Nivolumab, Pembrolizumab or Ipilimumab, or the combination of Nivolumab and Ipilimumab), the responder group had higher titers of antibodies specific for the tumor-associated antigens MDA (TRP1/TYRP1, TRP2/TYRP2, gp100, MelanA/MART1) and the Cancer-Testis antigen NY-ESO-1 at baseline, compared to non-responders. Serum analyses showed that NY-ESO-1, TRP1/TYRP1, and TRP2/TYRP2 specific antibodies consisted of several IgG subclasses while the IgG antibodies for MelanA were mostly IgG1 and the antibodies for gp100 were of the IgG2 isotype (75). Interestingly, none of these antibodies were IgG4, supporting the fact that IgG4 antibodies are not associated with a good prognosis in melanoma patients (67, 71, 75, 76).

In patients with metastatic melanoma who were treated with anti-CTLA-4 combined with anti-angiogenic VEGF-A-targeted treatment, there was an increase of antibody titers recognizing the immunoregulatory protein Galectin-1 (Gal-1). Anti-Gal-1 antibody titers in turn correlated with better disease outcome. Higher frequencies of complete or partial responses and improved overall survival were seen in these individuals. Gal-1 has been reported to be up-regulated in many tumor types including melanoma and is usually associated with poorer survival, contributing to tumor growth, angiogenesis, metastasis, and immune evasion (94).

Taken together, these results suggest that checkpoint blockade-associated restoration of T cell activity may also contribute to increasing the effectiveness of the humoral response and highlights the importance of the expressed antibodies as part of the anti-tumor activity. Furthermore, these studies suggest that melanoma-specific antibodies in pre-treatment sera may be promising indicators of CPI immunotherapy response and require further study.

CORRELATIONS WITH IMMUNE-RELATED ADVERSE EVENTS IN CPI THERAPY

Activation of immune responses as a result of checkpoint inhibition can lead to compromised immune self-tolerance and can consequently result in the development of irAEs, toxicities that mimic autoimmunity. The nature and frequency of irAEs vary among CPI agents and can include effects on the gastrointestinal tract (colitis), liver (hepatitis), lung (pneumonitis), and endocrine systems, including hypophysitis and insulin-dependent diabetes. The effects of anti-CTLA4 associated irAEs are generally more severe than those from anti PD-1 inhibition. Whether irAEs represent *de novo* events or whether they represent an unmasking of underlying immune mediated disease remains unclear (6).

Modulation of B cell phenotype has been correlated with irAE in patients undergoing CPI therapy. A recent study (described above) demonstrated an overall decline in B cell numbers but increased plasmablasts and $CD21^{lo}$ B cell subsets in patients

given combination checkpoint inhibition (**Table 1**) (77). These CPI-induced B cell changes positively correlated with frequency and severity of irAEs. Furthermore, patients with altered B cell populations were more likely to develop multi-organ immunotoxicity than those without, with these changes preceding toxicity by a median of 3 weeks.

B cells are proposed to contribute to autoimmunity *via* multiple mechanisms including as antibody secreting cells, cytokine producers, APCs, and immunoregulators. Given the important role of the checkpoint pathways in regulating immune homeostasis, it is not surprising that activation of the CTLA-4 pathway has been extensively studied in the treatment of autoimmunity. Specifically Abatecept, a fusion protein of CTLA-4 and IgG1 Fc portion (CTLA-4-Ig), is approved for abrogating immune overactivity in rheumatoid arthritis (95–97). CTLA-4-Ig is able to directly bind CD80/86; therefore, preventing CD28 binding and T cell co-stimulation. B cells are known to express CD80/86 and have been shown in mouse models and *in vitro* to be a direct target for CTLA-Ig. In synovial biopsy samples, CTLA-4-Ig has been shown to decrease B cell infiltrates (95). Humoral responses to T cell dependent and T cell independent antigenic stimulation are also suppressed by CTLA-4-Ig *in vivo* (98, 99). These insights from autoimmunity may also have relevance to our understanding of how CPI modification of B cells may induce irAE.

The presence of autoantibodies in CPI-induced irAE has been detected in a similar fashion to those seen in autoimmune disease in the absence of CPI treatment. For example some patients suffering with CPI-induced hypothyroidism have raised titers of anti-thyroperoxidase antibody and/or anti-thyrotropin receptor antibodies (100, 101). However, as these autoantibodies are not universally detected in CPI thyroid disorders, it is unclear whether they play a causative role. Similar observations have been made in a range of CPI-induced autoimmune phenomena including cases of myasthenia gravis (102), type 1 diabetes mellitus (103) and autoimmune hemolytic anemia (104). One study analyzed 23 common autoantibodies in baseline peripheral blood and post CPI treatment in 133 melanoma patients treated with Ipilimumab (105). This showed that 19% of patients developed autoantibodies on-treatment and a trend of association with onset of irAE which did not reach statistical significance. In this study, organ-specific autoantibodies did not correlate with irAE.

CPI-induced irAEs present a clinical challenge for patient selection and can lead to treatment discontinuation. Insights into the complex balance between activation and suppression of B cell subsets may infer consequences for both treatment response and irAE. In addition, the potential contributions of autoantibodies to irAE warrant further investigation and may be of direct relevance to B cell biology in the context of CPI.

HARNESSING THE HUMORAL IMMUNE RESPONSE IN MELANOMA IMMUNOTHERAPY

Data from several studies suggest that B cells can play multi-faceted roles in the immune response to melanoma and that melanoma-associated immune evasion mechanisms involve modulating B cell immunity in addition to suppressing T cell activation. Despite the impressive efficacy demonstrated by CPI, a large proportion of patients fail to respond and/or develop irAEs (4, 106, 107). Modulated B cell and antibody repertoires could either obstruct or facilitate the efficacy of CPI, depending on an array of factors in the TME. Further research identifying specific cellular surface markers and methods to clearly dissect the various B cell phenotypes will help improve our understanding of B cell immunity in melanoma and whether and how this is altered by CPI. This may then be exploited in clinical trials to help identify therapeutic strategies which can be combined with CPI to improve response. This may include the targeting of B cells; a pilot study using Rituximab (anti-CD20) to deplete B cells in melanoma patients increased the median time without recurrence from 6 to 42 months (108). On the other hand, B cell depletion therapy may have contrasting effects dependent on the stage of melanoma in mouse models (41). Given the newfound appreciation of the complexity of the TIL-B sub-populations, a more effective approach may involve depleting specific pro-tumor subsets, such as Bregs and IL-10+ B cells, while promoting anti-tumor subsets which could be combined with CPI as an adjuvant (24). PD1-PD-L1 engagement plays a role in suppressing B cell-mediated T cell activation, influencing CPI efficacy. Inhibition of PD-1 expressed on B cells may also be a mechanism through which CPI directly modulates B cell responses.

The targeting of the immunosuppressive TME cytokines IL-10, VEGF or TGF-β may effectively reinstate robust anti-tumor immunity by preventing IgG1 to IgG4 class switching and promoting anti-tumor B cell subsets to engage in tumor rejection. The hostile TME may be taken into consideration in the engineering of therapeutic antibody candidates, with the aim of potentially producing antibodies more resilient to immune suppression. CPI antibodies could additionally be engineered to have reduced binding to inhibitory Fc receptors, increased binding to activatory Fc receptors, or consider alternative isotypes such as IgE which has been implicated to improve immune surveillance in melanoma (109–111).

Aside from clinical response, irAEs are a major challenge of CPI in melanoma, as 96% of patients receiving combined CPI experienced at least one irAE in the CheckMate067 trial (4). Immune monitoring for early alterations in B cells following CPI may hold some promise in determining those at risk of developing irAEs. If confirmed to contribute to irAEs, therapies targeting plasmablasts and $CD21^{lo}$ B cells could be explored to reduce the risk. Serum and melanoma IgG4 and IgG4 + B cell levels may also be monitored, and therapies targeting these subsets could be conducted prior to CPI to establish a 'hot' tumor more likely to respond to CPI. Throughout CPI, B cell and antibody levels may be monitored to determine modulation and predict prognostic outcomes, and spikes may be countered through further depletion methods. Similar strategies could be conducted with immunosuppressive cytokines in the TME, anti-tumor B cell subsets and IgG1 levels.

CONCLUSION

It is increasingly appreciated that melanoma can develop intricate mechanisms to modulate the humoral immune response, and B cell immunity may also play a significant role in the efficacy and safety of CPI in melanoma. Taking all into consideration, it is highly conceivable that the promotion of anti-tumor B cell immunity with targeted therapies can also have clinical relevance in CPI response. Furthermore, immunomonitoring of humoral responses prior to, during, and following CPI in predicting irAEs should be explored. Given the complexity of tumor:immune cross-talk, utilizing B cells and antibodies as prognostic/predictive biomarkers in patient stratification and in immunomonitoring remains largely unexplored. Factoring in B cell immunity may harbor significant potential to overcome the two greatest challenges associated with CPI in melanoma: response and irAEs. Further studies in patients, *in vivo* and *ex vivo* models of immuno-oncology, alongside clinical testing, are required to fully comprehend and exploit likely multifaceted contributions.

AUTHOR CONTRIBUTIONS

ZNW, RJH, SC: Design and assembly of manuscript, design of illustrations. KH, HK, DT: Original draft writing. AnC, HJB, JC, AlC, RL, GO, RMH, MN, ST, LB: Planning, discussion and editing of the manuscript. JLG, AM-R, CH, JFS: Discussion, manuscript editing, clinical insights. SP, KEL, SNK: Manuscript design, project oversight. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Robbins HA, Clarke CA, Arron ST, Tatalovich Z, Kahn AR, Hernandez BY, et al. Melanoma Risk and Survival among Organ Transplant Recipients. *J Invest Dermatol* (2015) 135:2657–65. doi: 10.1038/jid.2015.312
2. Shiels MS, Copeland G, Goodman MT, Harrell J, Lynch CF, Pawlish K, et al. Cancer stage at diagnosis in patients infected with the human immunodeficiency virus and transplant recipients. *Cancer* (2015) 121:2063–71. doi: 10.1002/cncr.29324
3. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob J-J, Rutkowski P, Lao CD, et al. Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* (2019) 381:1535–46. doi: 10.1056/NEJMoa1910836
4. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob J-J, Rutkowski P, Cowey CL, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol* (2018) 19:1480–92. doi: 10.1016/S1470-2045(18)30700-9
5. FDA. https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-atezolizumab-braf-v600-unresectable-or-metastatic-melanoma.
6. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chávez A, Keegan N, Khamashta MA, et al. Immune-related adverse events of checkpoint inhibitors. *Nat Rev Dis Prim* (2020) 6(1):38. doi: 10.1038/s41572-020-0160-6
7. Alegre ML, Noel PJ, Eisfelder BJ, Chuang E, Clark MR, Reiner SL, et al. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. *J Immunol* (1996) 157:4762–70.
8. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* (2000) 192:303–10. doi: 10.1084/jem.192.2.303
9. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* (1994) 1:405–13. doi: 10.1016/1074-7613(94)90071-x
10. Sansom DM. CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology* (2000) 101:169–77. doi: 10.1046/j.1365-2567.2000.00121.x
11. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
12. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* (1996) 8:765–72. doi: 10.1093/intimm/8.5.765
13. Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, et al. PD-L2 Expression in Human Tumors: Relevance to Anti-PD-1 Therapy in Cancer. *Clin Cancer Res an Off J Am Assoc Cancer Res* (2017) 23:3158–67. doi: 10.1158/1078-0432.CCR-16-1761
14. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) 355:1428–33. doi: 10.1126/science.aaf1292
15. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci* (2012) 125:5591–6. doi: 10.1242/jcs.116392
16. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* (2012) 209:1201–17. doi: 10.1084/jem.20112741
17. Walker LSK, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. *Trends Immunol* (2015) 36:63–70. doi: 10.1016/j.it.2014.12.001
18. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* (2011) 332:600–3. doi: 10.1126/science.1202947
19. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Sci (80-)* (2015) 348:69–74. doi: 10.1126/science.aaa4971
20. Fu Q, Chen N, Ge C, Li R, Li Z, Zeng B, et al. Prognostic value of tumor-infiltrating lymphocytes in melanoma: a systematic review and meta-analysis. *Oncoimmunology* (2019) 8:1593806. doi: 10.1080/2162402X.2019.1593806

21. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* (2012) 12:298–306. doi: 10.1038/nrc3245
22. Selitsky SR, Mose LE, Smith CC, Chai S, Hoadley KA, Dittmer DP, et al. Prognostic value of B cells in cutaneous melanoma. *Genome Med* (2019) 11:1–11. doi: 10.1186/s13073-019-0647-5
23. Cursons J, Souza-Fonseca-Guimaraes F, Foroutan M, Anderson A, Hollande F, Hediyeh-Zadeh S, et al. A Gene Signature Predicting Natural Killer Cell Infiltration and Improved Survival in Melanoma Patients. *Cancer Immunol Res* (2019) 7:1162–74. doi: 10.1158/2326-6066.CIR-18-0500
24. Chiaruttini G, Mele S, Opzoomer J, Crescioli S, Ilieva KM, Lacy KE, et al. B cells and the humoral response in melanoma: The overlooked players of the tumor microenvironment. *Oncoimmunology* (2017) 6:1–11. doi: 10.1080/2162402X.2017.1294296
25. Germain C, Gnjatic S, Dieu-Nosjean M-C. Tertiary Lymphoid Structure-Associated B Cells are Key Players in Anti-Tumor Immunity. *Front Immunol* (2015) 6:67. doi: 10.3389/fimmu.2015.00067
26. Cipponi A, Mercier M, Seremet T, Baurain J-F, Theate I, van den Oord J, et al. Neogenesis of lymphoid structures and antibody responses occur in human melanoma metastases. *Cancer Res* (2012) 72:3997–4007. doi: 10.1158/0008-5472.CAN-12-1377
27. Cabrita R, Lauss M, Sanna A, Donia M, Skaarup Larsen M, Mitra S, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature* (2020) 577:561–5. doi: 10.1038/s41586-019-1914-8
28. Munoz-Erazo L, Rhodes JL, Marion VC, Kemp RA. Tertiary lymphoid structures in cancer – considerations for patient prognosis. *Cell Mol Immunol* (2020) 17:570–5. doi: 10.1038/s41423-020-0457-0
29. Helmink BA, Reddy SM, Gao J, Zhang S, Basar R, Thakur R, et al. B cells and tertiary lymphoid structures promote immunotherapy response. *Nature* (2020) 577(7791):549–55. doi: 10.1038/s41586-019-1922-8
30. Ladányi A. Prognostic and predictive significance of immune cells infiltrating cutaneous melanoma. *Pigment Cell Melanoma Res* (2015) 28:490–500. doi: 10.1111/pcmr.12371
31. Hussein MR, Elsers DAH, Fadel SA, Omar AEM. Immunohistological characterisation of tumour infiltrating lymphocytes in melanocytic skin lesions. *J Clin Pathol* (2006) 59:316–24. doi: 10.1136/jcp.2005.028860
32. Hillen F, Baeten CIM, Van De Winkel A, Creytens D, Van Der Schaft DWJ, Winnepenninckx V, et al. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. *Cancer Immunol Immunother* (2008) 57:97–106. doi: 10.1007/s00262-007-0353-9
33. Martinez-Rodriguez M, Thompson AK, Monteagudo C. A significant percentage of CD20-positive TILs correlates with poor prognosis in patients with primary cutaneous malignant melanoma. *Histopathology* (2014) 65:726–8. doi: 10.1111/his.12437
34. Karagiannis P, Gilbert AE, Nestle FO, Karagiannis SN. IgG4 antibodies and cancer-associated inflammation. *Oncoimmunology* (2013) 2:e24889. doi: 10.4161/onci.24889
35. Saul L, Ilieva KM, Bax HJ, Karagiannis P, Correa I, Rodriguez-Hernandez I, et al. IgG subclass switching and clonal expansion in cutaneous melanoma and normal skin. *Sci Rep* (2016) 6:1–12. doi: 10.1038/srep29736
36. Gilbert AE, Karagiannis P, Dodev T, Koers A, Lacy K, Josephs DH, et al. Monitoring the systemic human memory B cell compartment of melanoma patients for anti-tumor IgG antibodies. *PLoS One* (2011) 6:e19330. doi: 10.1371/journal.pone.0019330
37. Bosisio FM, Wilmott JS, Volders N, Mercier M, Wouters J, Stas M, et al. Plasma cells in primary melanoma. Prognostic significance and possible role of IgA. *Mod Pathol* (2016) 29:347–58. doi: 10.1038/modpathol.2016.28
38. Kim H, Hong SH, Kim JY, Kim IC, Park YW, Lee SJ, et al. Preclinical development of a humanized neutralizing antibody targeting HGF. *Exp Mol Med* (2017) 49:e309–9. doi: 10.1038/emm.2017.21
39. Rodríguez-Pinto D. B cells as antigen presenting cells. *Cell Immunol* (2005) 238:67–75. doi: 10.1016/j.cellimm.2006.02.005
40. Lund FE. Cytokine-producing B lymphocytes - key regulators of immunity. *Curr Opin Immunol* (2008) 20:332–8. doi: 10.1016/j.coi.2008.03.003
41. Griss J, Bauer W, Wagner C, Simon M, Chen M, Grabmeier-Pfistershammer K, et al. B cells sustain inflammation and predict response to immune checkpoint blockade in human melanoma. *Nat Commun* (2019) 10(1):4186. doi: 10.1038/s41467-019-12160-2
42. Endres R, Alimzhanov MB, Plitz T, Fütterer A, Kosco-Vilbois MH, Nedospasov SA, et al. Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. *J Exp Med* (1999) 189:159–68. doi: 10.1084/jem.189.1.159
43. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res* (2012) 72:1070–80. doi: 10.1158/0008-5472.CAN-11-3218
44. Garg K, Maurer M, Griss J, Brüggen MC, Wolf IH, Wagner C, et al. Tumor-associated B cells in cutaneous primary melanoma and improved clinical outcome. *Hum Pathol* (2016) 54:157–64. doi: 10.1016/j.humpath.2016.03.022
45. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* (2010) 32:129–40. doi: 10.1016/j.immuni.2009.11.009
46. Ashour HM, Seif TM. The role of B cells in the induction of peripheral T cell tolerance. *J Leukoc Biol* (2007) 82:1033–9. doi: 10.1189/jlb.0507310
47. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* (2002) 16:219–30. doi: 10.1016/S1074-7613(02)00274-1
48. Duddy ME, Alter A, Bar-Or A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *J Immunol* (2004) 172:3422–7. doi: 10.4049/jimmunol.172.6.3422
49. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* (2013) 5:173ra23. doi: 10.1126/scitranslmed.3005407
50. Wu H, Su Z, Barnie PA. The role of B regulatory (B10) cells in inflammatory disorders and their potential as therapeutic targets. *Int Immunopharmacol* (2020) 78:106111. doi: 10.1016/j.intimp.2019.106111
51. Inoue S, Leitner WW, Golding B, Scott D. Inhibitory effects of B cells on antitumor immunity. *Cancer Res* (2006) 66:7741–7. doi: 10.1158/0008-5472.CAN-05-3766
52. Wei B, Velazquez P, Turovskaya O, Spricher K, Aranda R, Kronenberg M, et al. Mesenteric B cells centrally inhibit CD4+ T cell colitis through interaction with regulatory T cell subsets. *Proc Natl Acad Sci USA* (2005) 102:2010–5. doi: 10.1073/pnas.0409449102
53. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* (2010) 17:121–34. doi: 10.1016/j.ccr.2009.12.019
54. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, et al. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4+ T cells to T-regulatory cells. *Cancer Res* (2011) 71:3505–15. doi: 10.1158/0008-5472.CAN-10-4316
55. Wejksza K, Lee-Chang C, Bodogai M, Bonzo J, Gonzalez FJ, Lehrmann E, et al. Cancer-Produced Metabolites of 5-Lipoxygenase Induce Tumor-Evoked Regulatory B Cells via Peroxisome Proliferator–Activated Receptor α. *J Immunol* (2013) 190:2575–84. doi: 10.4049/jimmunol.1201920
56. Han S, Feng S, Ren M, Ma E, Wang X, Xu L, et al. Glioma cell-derived placental growth factor induces regulatory B cells. *Int J Biochem Cell Biol* (2014) 57:63–8. doi: 10.1016/j.biocel.2014.10.005
57. Zhang Y, Gallastegui N, Rosenblatt JD. Regulatory B cells in anti-tumor immunity. *Int Immunol* (2015) 27:521–30. doi: 10.1093/intimm/dxv034
58. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. *J Clin Invest* (2011) 121:4268–80. doi: 10.1172/JCI59266
59. Lindner S, Dahlke K, Sontheimer K, Hagn M, Kaltenmeier C, Barth TFE, et al. Interleukin 21-induced granzyme b-expressing b cells infiltrate tumors and regulate t cells. *Cancer Res* (2013) 73:2468–79. doi: 10.1158/0008-5472.CAN-12-3450
60. Wang W, Yuan X, Chen H, Xie G, Ma Y, Zheng Y, et al. CD19 +CD24hiCD38hi Bregs involved in downregulate helper T cells and

upregulate regulatory T cells in gastric cancer. *Oncotarget* (2015) 6:33486–99. doi: 10.18632/oncotarget.5588
61. Kobayashi T, Oishi K, Okamura A, Maeda S, Komuro A, Hamaguchi Y, et al. Regulatory B1a Cells Suppress Melanoma Tumor Immunity via IL-10 Production and Inhibiting T Helper Type 1 Cytokine Production in Tumor-Infiltrating CD8+ T Cells. *J Invest Dermatol* (2019) 139:1535–44.e1. doi: 10.1016/j.jid.2019.02.016
62. Schioppa T, Moore R, Thompson RG, Rosser EC, Kulbe H, Nedospasov S, et al. B regulatory cells and the tumor-promoting actions of TNF-α during squamous carcinogenesis. *Proc Natl Acad Sci USA* (2011) 108:10662–7. doi: 10.1073/pnas.1100994108
63. Yanaba K, Kamata M, Ishiura N, Shibata S, Asano Y, Tada Y, et al. Regulatory B cells suppress imiquimod-induced, psoriasis-like skin inflammation. *J Leukoc Biol* (2013) 94:563–73. doi: 10.1189/jlb.1112562
64. Ellyard JI, Simson L, Parish CR. Th2-mediated anti-tumour immunity: friend or foe? *Tissue Antigens* (2007) 70:1–11. doi: 10.1111/j.1399-0039.2007.00869.x
65. Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* (1991) 77:1859–70. doi: 10.1182/blood.V77.9.1859.bloodjournal7791859
66. Satoguina JS, Weyand E, Larbi J, Hoerauf A. T Regulatory-1 Cells Induce IgG4 Production by B Cells: Role of IL-10. *J Immunol* (2005) 174:4718–26. doi: 10.4049/jimmunol.174.8.4718
67. Karagiannis P, Gilbert AE, Josephs DH, Ali N, Dodev T, Saul L, et al. IgG4 subclass antibodies impair antitumor immunity in melanoma. *J Clin Invest* (2013) 123:1457–74. doi: 10.1172/JCI65579
68. Daveau M, Pavie-Fischer J, Rivat L, Rivat C, Ropartz C, Peter HH, et al. IgG4 subclass in malignant melanoma. *J Natl Cancer Inst* (1977) 58:189–92. doi: 10.1093/jnci/58.2.189
69. Aalberse RC, Schuurman J. IgG4 breaking the rules. *Immunology* (2002) 105:9–19. doi: 10.1046/j.0019-2805.2001.01341.x
70. Wang H, Xu Q, Zhao C, Zhu Z, Zhu X, Zhou J, et al. An immune evasion mechanism with IgG4 playing an essential role in cancer and implication for immunotherapy. *J Immunother Cancer* (2020) 8:e000661. doi: 10.1136/jitc-2020-000661
71. Karagiannis P, Villanova F, Josephs DH, Correa I, Van Hemelrijck M, Hobbs C, et al. Elevated IgG4 in patient circulation is associated with the risk of disease progression in melanoma. *Oncoimmunology* (2015) 4:e1032492–e1032492. doi: 10.1080/2162402X.2015.1032492
72. Crescioli S, Correa I, Karagiannis P, Davies AM, Sutton BJ, Nestle FO, et al. IgG4 Characteristics and Functions in Cancer Immunity. *Curr Allergy Asthma Rep* (2016) 16:7. doi: 10.1007/s11882-015-0580-7
73. van de Veen W, Globinska A, Jansen K, Straumann A, Kubo T, Verschoor D, et al. A novel proangiogenic B cell subset is increased in cancer and chronic inflammation. *Sci Adv* (2020) 6:eaaz3559. doi: 10.1126/sciadv.aaz3559
74. DeFalco J, Harbell M, Manning-Bog A, Baia G, Scholz A, Millare B, et al. Non-progressing cancer patients have persistent B cell responses expressing shared antibody paratopes that target public tumor antigens. *Clin Immunol* (2018) 187:37–45. doi: 10.1016/j.clim.2017.10.002
75. Fässler M, Diem S, Mangana J, Hasan Ali O, Berner F, Bomze D, et al. Antibodies as biomarker candidates for response and survival to checkpoint inhibitors in melanoma patients. *J Immunother Cancer* (2019) 7(1):50. doi: 10.1186/s40425-019-0523-2
76. Diem S, Fässler M, Bomze D, Ali OH, Berner F, Niederer R, et al. Immunoglobulin G and Subclasses as Potential Biomarkers in Metastatic Melanoma Patients Starting Checkpoint Inhibitor Treatment. *J Immunother* (2019) 42:89–93. doi: 10.1097/CJI.0000000000000255
77. Das R, Bar N, Ferreira M, Newman AM, Zhang L, Bailur JK, et al. Early b cell changes predict autoimmunity following combination immune checkpoint blockade. *J Clin Invest* (2018) 128:715–20. doi: 10.1172/JCI96798
78. Amaria RN, Reddy SM, Tawbi HA, Davies MA, Ross MI, Glitza IC, et al. Neoadjuvant immune checkpoint blockade in high-risk resectable melanoma. *Nat Med* (2018) 24:1649–54. doi: 10.1038/s41591-018-0197-1
79. Kuehn HS, Ouyang W, Lo B, Deenick EK, Niemela JE, Avery DT, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* (2014) 345:1623–7. doi: 10.1126/science.1255904
80. Petitprez F, de Reyniès A, Keung EZ, Chen TW-W, Sun C-M, Calderaro J, et al. B cells are associated with survival and immunotherapy response in sarcoma. *Nature* (2020) 577(7791):556–60. doi: 10.1038/s41586-019-1906-8
81. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. *Nat Rev Cancer* (2012) 12:278–87. doi: 10.1038/nrc3236
82. Sharonov GV, Serebrovskaya EO, Yuzhakova DV, Britanova OV, Chudakov DM. B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nat Rev Immunol* (2020) 20:294–307. doi: 10.1038/s41577-019-0257-x
83. Shi J-Y, Gao Q, Wang Z-C, Zhou J, Wang X-Y, Min Z-H, et al. Margin-infiltrating CD20(+) B cells display an atypical memory phenotype and correlate with favorable prognosis in hepatocellular carcinoma. *Clin Cancer Res an Off J Am Assoc Cancer Res* (2013) 19:5994–6005. doi: 10.1158/1078-0432.CCR-12-3497
84. Hamanaka Y, Suehiro Y, Fukui M, Shikichi K, Imai K, Hinoda Y. Circulating anti-MUC1 IgG antibodies as a favorable prognostic factor for pancreatic cancer. *Int J Cancer* (2003) 103:97–100. doi: 10.1002/ijc.10801
85. Kurtenkov O, Klaamas K, Mensdorff-Pouilly S, Miljukhina L, Shljapnikova L, Chužmarov V. Humoral immune response to MUC1 and to the Thomsen-Friedenreich (TF) glycotope in patients with gastric cancer: Relation to survival. *Acta Oncol (Madr)* (2007) 46:316–23. doi: 10.1080/02841860601055441
86. Fremd C, Stefanovic S, Beckhove P, Pritsch M, Lim H, Wallwiener M, et al. Mucin 1-specific B cell immune responses and their impact on overall survival in breast cancer patients. *Oncoimmunology* (2015) 5:e1057387–e1057387. doi: 10.1080/2162402X.2015.1057387
87. Montfort A, Pearce O, Maniati E, Vincent BG, Bixby L, Böhm S, et al. A Strong B-cell Response Is Part of the Immune Landscape in Human High-Grade Serous Ovarian Metastases. *Clin Cancer Res an Off J Am Assoc Cancer Res* (2017) 23:250–62. doi: 10.1158/1078-0432.CCR-16-0081
88. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med* (2000) 6:443–6. doi: 10.1038/74704
89. Cohen-Solal JFG, Cassard L, Fournier EM, Loncar SM, Fridman WH, Sautès-Fridman C. Metastatic melanomas express inhibitory low affinity fc gamma receptor and escape humoral immunity. *Dermatol Res Pract* (2010) 2010:657406. doi: 10.1155/2010/657406
90. Bolotin DA, Poslavsky S, Davydov AN, Frenkel FE, Fanchi L, Zolotareva OI, et al. Antigen receptor repertoire profiling from RNA-seq data. *Nat Biotechnol* (2017) 35:908–11. doi: 10.1038/nbt.3979
91. Nicholas KJ, Zern EK, Barnett L, Smith RM, Lorey SL, Copeland CA, et al. B cell responses to HIV antigen are a potent correlate of viremia in HIV-1 infection and improve with PD-1 blockade. *PloS One* (2013) 8:e84185. doi: 10.1371/journal.pone.0084185
92. Ohaegbulam KC, Assal A, Lazar-Molnar E, Yao Y, Zang X. Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway. *Trends Mol Med* (2015) 21:24–33. doi: 10.1016/j.molmed.2014.10.009
93. Nguyen N, Mamchak A, Severgnini M, Williamson KS, Wu X, Drabek EF, et al. Abstract 615: Increased somatic hypermutation in the immunoglobulin sequences of melanoma patients who have durable response to checkpoint inhibitor therapy. *Proceedings of the American Association for Cancer Research Annual Meeting 2018*. Chicago, IL. Philadelphia (PA): AACR; *Cancer Res* (2018) 78(13 Suppl). doi: 10.1158/1538-7445.am2018-615
94. Wu X, Li J, Connolly EM, Liao X, Ouyang J, Giobbie-Hurder A, et al. Combined Anti-VEGF and Anti-CTLA-4 Therapy Elicits Humoral Immunity to Galectin-1 Which Is Associated with Favorable Clinical Outcomes. *Cancer Immunol Res* (2017) 5:446–54. doi: 10.1158/2326-6066.CIR-16-0385
95. Buch MH, Boyle DL, Rosengren S, Saleem B, Reece RJ, Rhodes LA, et al. Mode of action of abatacept in rheumatoid arthritis patients having failed tumour necrosis factor blockade: A histological, gene expression and dynamic magnetic resonance imaging pilot study. *Ann Rheum Dis* (2009) 68:1220–7. doi: 10.1136/ard.2008.091876
96. Genovese MC, Becker J-C, Schiff M, Luggen M, Sherrer Y, Kremer J, et al. Abatacept for Rheumatoid Arthritis Refractory to Tumor Necrosis Factor α Inhibition. *N Engl J Med* (2005) 353:1114–23. doi: 10.1056/nejmoa050524
97. Kremer JM, Genant HK, Moreland LW, Russell AS, Emery P, Abud-Mendoza C, et al. Effects of abatacept in patients with methotrexate-

resistant active rheumatoid arthritis: A randomized trial. *Ann Intern Med* (2006) 144:865–76. doi: 10.7326/0003-4819-144-12-200606200-00003
98. Linsley PS, Wallace PM, Johnson J, Gibson MG, Greene JAL, Ledbetter JA, et al. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Sci (80-)* (1992) 257:792–5. doi: 10.1126/science.1496399
99. Tay L, Leon F, Vratsanos G, Raymond R, Corbo M. Vaccination response to tetanus toxoid and 23-valent pneumococcal vaccines following administration of a single dose of abatacept: A randomized, open-label, parallel group study in healthy subjects. *Arthritis Res Ther* (2007) 9(2):R38. doi: 10.1186/ar2174
100. Osorio JC, Ni A, Chaft JE, Pollina R, Kasler MK, Stephens D, et al. Antibody-mediated thyroid dysfunction during T-cell checkpoint blockade in patients with non-small-cell lung cancer. *Ann Oncol* (2017) 28:583–9. doi: 10.1093/annonc/mdw640
101. Kobayashi T, Iwama S, Yasuda Y, Okada N, Tsunekawa T, Onoue T, et al. Patients with antithyroid antibodies are prone to develop destructive thyroiditis by nivolumab: A prospective study. *J Endocr Soc* (2018) 2:241–51. doi: 10.1210/JS.2017-00432
102. Shirai T, Sano T, Kamijo F, Saito N, Miyake T, Kodaira M, et al. Acetylcholine receptor binding antibody-associated myasthenia gravis and rhabdomyolysis induced by nivolumab in a patient with melanoma. *Jpn J Clin Oncol* (2016) 46:86–8. doi: 10.1093/jjco/hyv158
103. Stamatouli AM, Quandt Z, Perdigoto AL, Clark PL, Kluger H, Weiss SA, et al. Collateral Damage: Insulin-Dependent Diabetes Induced With Checkpoint Inhibitors. *Diabetes* 67(8):1471–80. doi: 10.2337/dbi18-0002
104. Kong BY, Micklethwaite KP, Swaminathan S, Kefford RF, Carlino MS. Autoimmune hemolytic anemia induced by anti-PD-1 therapy in metastatic melanoma. *Melanoma Res* (2016) 26:202–4. doi: 10.1097/CMR.0000000000000232
105. De Moel EC, Rozeman EA, Kapiteijn EH, Verdegaal EME, Grummels A, Bakker JA, et al. Autoantibody development under treatment with immune-checkpoint inhibitors. *Cancer Immunol Res* (2019) 7:6–11. doi: 10.1158/2326-6066.CIR-18-0245
106. Cogdill AP, Andrews MC, Wargo JA. Hallmarks of response to immune checkpoint blockade. *Br J Cancer* (2017) 117:1–7. doi: 10.1038/bjc.2017.136
107. Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. *Br J Cancer* (2018) 118:9–16. doi: 10.1038/bjc.2017.434
108. Pinc A, Somasundaram R, Wagner C, Hörmann M, Karanikas G, Jalili A, et al. Targeting CD20 in melanoma patients at high risk of disease recurrence. *Mol Ther* (2012) 20:1056–62. doi: 10.1038/mt.2012.27
109. Josephs DH, Spicer JF, Karagiannis P, Gould HJ, Karagiannis SN. IgE immunotherapy: a novel concept with promise for the treatment of cancer. *MAbs* (2014) 6:54–72. doi: 10.4161/mabs.27029
110. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* (2013) 210:1695–710. doi: 10.1084/jem.20130579
111. Quezada SA, Peggs KS. Lost in translation: Deciphering the mechanism of action of anti-human CTLA-4. *Clin Cancer Res* (2019) 25:1130–2. doi: 10.1158/1078-0432.CCR-18-2509

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The Origin of B-cells: Human Fetal B Cell Development and Implications for the Pathogenesis of Childhood Acute Lymphoblastic Leukemia

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Human B-lymphopoiesis is a dynamic life-long process that starts *in utero* by around six post-conception weeks. A detailed understanding of human fetal B-lymphopoiesis and how it changes in postnatal life is vital for building a complete picture of normal B-lymphoid development through ontogeny, and its relevance in disease. B-cell acute lymphoblastic leukemia (B-ALL) is one of the most common cancers in children, with many of the leukemia-initiating events originating *in utero*. It is likely that the biology of B-ALL, including leukemia initiation, maintenance and progression depends on the developmental stage and type of B-lymphoid cell in which it originates. This is particularly important for early life leukemias, where specific characteristics of fetal B-cells might be key to determining how the disease behaves, including response to treatment. These cellular, molecular and/or epigenetic features are likely to change with age in a cell intrinsic and/or microenvironment directed manner. Most of our understanding of fetal B-lymphopoiesis has been based on murine data, but many recent studies have focussed on characterizing human fetal B-cell development, including functional and molecular assays at a single cell level. In this mini-review we will give a short overview of the recent advances in the understanding of human fetal B-lymphopoiesis, including its relevance to infant/childhood leukemia, and highlight future questions in the field.

Keywords: B-lymphopoiesis, human fetal, childhood, infant, leukemia, B-ALL, B-cell

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INTRODUCTION

Unraveling the details of human hematopoietic development during embryogenesis is crucial for both basic and medical science. Relative contributions of different progenitor compartments and downstream lineage specificity vary during human ontogeny. Detailed immunophenotyping of fetal hematopoietic tissues from 6 to 20 weeks post conception (pcw) has identified that a much higher proportion of fetal bone marrow (FBM) cells are B-lymphoid than fetal liver (FL) and adult bone marrow (ABM) (1). In keeping with this, the changing lymphoid/myeloid specification in aging bone marrow has been described (2–4). Secondly, a switch from multipotent to largely oligo/unipotent stem cells is also known to occur between fetal and adult life (5). Thirdly, differences in the proliferative capacity of human fetal and postnatal hematopoietic stem and progenitor cells (HSPC) have been demonstrated using functional and molecular studies, with a marked and progressive increase in stem cell quiescence evident during physiological aging

(6–9). In addition, some fetal gene expression programs are inherently oncogenic (10–12), and high mutation rates are seen both in hematopoietic and non-hematopoietic fetal stem cells when compared to postnatal tissues (13, 14). Therefore, understanding how hematopoiesis changes through human ontogeny is crucial if we are to understand the site- and stage-specific variation in HSPC throughout the human lifetime and the role it plays in hematological disorders/diseases.

Fetal hematopoiesis is of particular interest in understanding childhood blood disorders that originate before birth. Significantly all infant leukemia and much of childhood acute lymphoblastic leukemia (ALL) originate before birth (15, 16).

ALL is the most common childhood malignancy, and 80% of childhood-ALL are of the B-lymphoid lineage. Early onset B-ALL can be divided into infant ALL (iALL) presenting at age <12 months or childhood-ALL presenting at age >12 months. While outcomes for childhood-ALL have improved dramatically over the past few years to reach an overall survival (OS) rate of >90% (17); the OS rate is only ∼60% in infants (18). The reasons for such disparate outcomes is not clear, but the clues might lie in the developmental origins of infant and childhood-ALL.

Advances in understanding fetal hematopoiesis and prenatal oncogenic events, have been limited by a number of factors. The scarcity of human fetal biological samples is compounded by the difficulty in working with very small numbers of HSPC that can be obtained from each sample. Thus, majority of our understanding of early hematopoiesis development has come from murine studies. Neither these, nor adult human models can be used as a faithful surrogate for human fetal hematopoiesis (5, 19, 20). This in turn leads to difficulties in making developmentally relevant model systems for human leukemia (21, 22).

In this review we will focus on recent advances in our understanding of human B-lymphopoiesis during ontogeny, especially in fetal life, and review progenitor compartments therein which may align to the origin of iALL and childhood-ALL.

HUMAN B-LYMPHOPOIESIS

Hematopoiesis has traditionally been described as a hierarchical process with hematopoietic stem cells (HSCs) at the apex; these divide and differentiate into progressively restricted progenitors that subsequently give rise to the mature cell types of the hematopoietic and immune system (23, 24).

The traditional human B-lymphoid developmental hierarchy in adult life demonstrates the following lineage progression in ABM: HSC, multi-potent progenitors (MPP), lymphoid-primed multi-potent progenitors (LMPP) (25, 26), multi-lymphoid progenitors (MLP) (27, 28), common lymphoid progenitors (CLP) (29), ProB-progenitors, PreB-cells and finally mature B-cells (30–32) (**Figure 1**). Lineage commitment is a multi-stage process defined by transcription factors and their related gene regulatory networks, influenced both by cell intrinsic factors and extracellular signals from the microenvironment (29, 33–35). CD19 expression is the hallmark of B-lineage commitment, with ProB-progenitors being the first $CD19^+$ cells in ABM that also initiate immunoglobulin heavy chain V_H-D_H-J_H rearrangement (31, 36). In recent years, single cell approaches have been extensively applied to delineate cellular hierarchies and molecular pathways in hematopoiesis (37, 38). However, the majority of studies have been done in human cord blood (5, 39) or adult tissues (38, 40, 41).

Recent studies have begun to leverage sophisticated transcriptomic and functional assays to identify B-lymphoid progenitor compartments in the fetus that are not represented in the adult. These, and/or their microenvironment, are hypothesized to be important for the pathogenesis of infant and childhood leukemias, and perhaps also adult malignancies with *in utero* origins (15, 42).

HUMAN FETAL B-LYMPHOPOIESIS

The timings and sites of fetal hematopoiesis have been broadly mapped out in humans. Hematopoiesis is initiated at day 18 post conception in the yolk sac, independently definitive HSC emerge from the aorta-gonad-mesonephros (AGM) at 4 pcw and subsequently migrate to the FL and then bone marrow, which remains the main site of hematopoiesis after birth (43–47). HSCs colonize the FL from 5th pcw, and they are detectable later in the long bones at 10–12 pcw (1, 48).

In humans, the first evidence of onset of embryonic lymphopoiesis is in the FL at 6 pcw, with multi-potent progenitors (HSC, MPP, LMPP) and fetal-specific oligo-potent early lymphoid progenitors (ELP) detectable. B-progenitors and B-cells are seen in FL by 7 pcw (9, 30, 49, 50). From 2nd trimester the FBM takes over from the FL as the main site of B-lymphopoiesis (1, 51).

Fetal Lymphoid Progenitors

Interestingly, in murine models immune restricted cells with lymphoid potential are observed in the yolk sac (YS), preceding the first HSCs found in FL; these have potential to produce lymphocytes and granulocyte macrophage progenitors (52) and express Il7 receptor (Il7-r/CD127). Transcriptomic data suggests that such lymphoid progenitors may also be present in human YS (9) but these have not been systematically characterized yet. In humans a potentially analogous cell has been identified in the FL, from 6 pcw; ($CD34^+CD19^-IL7R^+$) (1, 50, 53, 54). Similar $IL7R^+$ progenitors have been described in human FBM (1). FL and FBM $CD34^+CD127^+CD19^-CD10^-$ ELP have been characterized by functional and transcriptomic assays, and shown to generate B, T and NK cells while retaining some residual myeloid output. These fetal-specific ELP are very rare in postnatal life (1, 54). There has therefore, been considerable interest in these cells as potential target cells for childhood-ALL.

Fetal B-Progenitors

From 7 pcw the presence of two committed $CD19^+$ B-progenitors downstream of ELP has been confirmed in human FL samples; PreProB ($CD34^+CD19^+CD10^-$) and ProB ($CD34^+CD19^+CD10^+$) progenitors; differing in their CD10

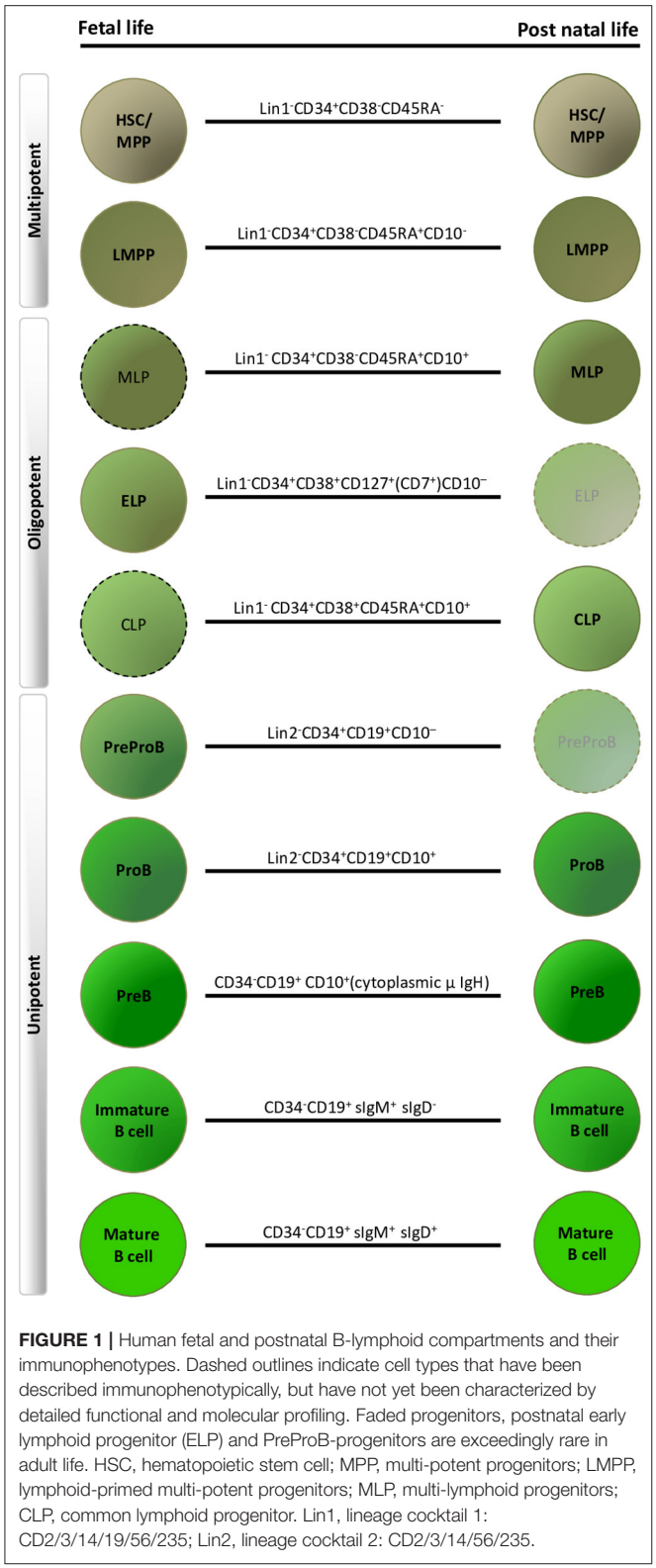

FIGURE 1 | Human fetal and postnatal B-lymphoid compartments and their immunophenotypes. Dashed outlines indicate cell types that have been described immunophenotypically, but have not yet been characterized by detailed functional and molecular profiling. Faded progenitors, postnatal early lymphoid progenitor (ELP) and PreProB-progenitors are exceedingly rare in adult life. HSC, hematopoietic stem cell; MPP, multi-potent progenitors; LMPP, lymphoid-primed multi-potent progenitors; MLP, multi-lymphoid progenitors; CLP, common lymphoid progenitor. Lin1, lineage cocktail 1: CD2/3/14/19/56/235; Lin2, lineage cocktail 2: CD2/3/14/56/235.

expression (1, 50, 54). Similar progenitors have been described in cord blood (55, 56). PreProB-progenitors account for ∼2.5% and ProB-progenitors ∼8% of FL $CD34^+$ cells, and these frequencies remain fairly stable in FL between 7 and 20 pcw. These cells have also been identified by single cell transcriptomic approaches in the human FL (9).

PreProB and ProB-progenitors are also present and markedly expanded in human FBM (1). Both B-progenitor compartments undergo marked expansion in the early stages of colonization of FBM, to account for up to around 20% and 11% of FBM $CD34^+$ cells, respectively, at 11 pcw. Later in the second trimester PreProB-progenitors plateau while ProB-progenitors expand further to >30% of $CD34^+$ cells in FBM. By contrast, ABM $CD34^+$ compartment was found to have only 0.5% PreProB-progenitors and 14% ProB-progenitors (1).

Both PreProB and ProB-progenitors lie downstream of ELP and generate exclusively B-lymphoid progeny *in vitro* and *in vivo*. Functional and molecular studies have established that FBM PreProB-progenitors lie upstream of ProB-progenitors, and are therefore the earliest B-lymphoid restricted progenitors in the fetal B-cell developmental hierarchy (1).

B cell maturation, defined by B cell receptor diversification, commences in B-lymphoid progenitors in fetal life. Fetal ELP and PreProB-progenitors show partial (D_H-J_H) IgH rearrangement (1, 54), whereas the more mature ProB-progenitors demonstrate complete V_H-D_H-J_H rearrangement (1).

Fetal B-Cells

$CD19^+$ B-cells have been reported in FL and FBM by many groups (30, 48, 49, 57–59), and recently been characterized in greater detail (1, 9, 60, 61). Evidence of B cell maturation is demonstrable in human fetal life, with polyclonal $CD19^+IgM^+$ B-cells (60–63). Although FL and FBM immunoglobulin heavy chain repertoires are equally diversified, FL appears to be the main source of IgM natural immunity during the 2nd trimester, and this correlates with the majority of B-cells in 2nd trimester FBM being $CD34^-CD19^+CD10^+IgM/D^-$ PreB-cells with a relative lack of more downstream immature and transitional B-cells (60).

B1 B-Cells and their Putative Progenitors

B cells can be further divided into B1 B-cells of the innate immune system and "conventional" B2 B-cells of the adaptive immune system. This division is well-established in mice, where $sIgM^+CD11b^+CD5^+$ B1a B-cells were first identified (64, 65) through the search for, the still elusive, cell of origin of adult human CLL (42, 66). B1b B-cells ($sIgM^+CD11b^+CD5^-$) were subsequently described (67); both these subtypes are seen predominantly in serous cavities. Further characterization of splenic B1 cells have identified them to be $CD5^{+/-}CD19^{hi}CD1d^{mid}CD23^-CD43^+IgM^{hi}IgD^{lo}$ (68). Murine B1 B-cell progenitors are found in the yolk sac (69) prior to the emergence of the first definitive HSCs in the FL, which have both B1 and B2 B-cell output (70). The B-cell output skews toward B2 B-cells over ontogeny, with B1 B-cell output being exceedingly rare in ABM (65, 71).

Human B1 B-cells and their upstream progenitors have been proposed as the *in utero* cell of origin for infant and childhood-ALL (72) and as having a role in auto-immune disease (73, 74). In humans, B1 B-cells were described in

umbilical cord blood and adult peripheral blood. These cells were $CD20^{+}CD27^{+}CD43^{+}CD38^{lo/int}$ and functioned in line with murine counterparts, including spontaneous IgM secretion, constitutional BCR receptor activity and ability to induce allogeneic T cell proliferation (75). Putative B1 B-cells have also been described in human fetal hematopoiesis, with greatest frequencies in 10 pcw FL, decreasing as FBM is colonized (59). After birth, estimates of B1 B-cell populations range from 1 to 10% circulating B-cells, this frequency falls as age increases (76–78).

The progenitors of B1 B-cells in humans remain elusive and contentious. Two theories posit either a lineage (or layered) model where different subtypes arise from different progenitors or a selection model whereby there is interconversion between B1 and B2 B-cells. In humans, CD27 (one of the cell surface markers of B1 B-cells) expression in ABM ProB-cells coincides with *LIN28B* expression levels similar to that seen in FL. These cells mature preferentially to B1-like B-cells compared to their $CD27^{-}$ counterparts. It is not clear whether this relates to a separate lineage or alternative differentiation potential (79).

In summary, human fetal B-lymphopoiesis starts around 6 pcw in FL, with B-cell production happening simultaneously in FL and FBM from 2nd trimester. Hematopoiesis in the FBM is skewed toward B-lymphopoiesis in 2nd trimester. In addition there are fetal-specific B-lymphoid progenitors (ELP and PreProB-progenitors), B-cells (B1 B-cells) and developmental pathways that are different from human adult life (**Figure 1**).

MOLECULAR PROFILE OF FETAL B CELL PROGENITORS

Recent studies suggest that the ontogenic switch of B1 to B2 B-cells in murine B-cell lineage fate of progenitor cells is determined by a combination of intrinsic fetal gene expression programs (*Lin28b*) (80) and extrinsic FL environmental factors (81). Whole transcriptome profiling of murine fetal and adult B cell progenitors showed distinct differences between B-1 and B-2 B-cells as well as between fetal and adult progenitors (82). Although it is well-accepted that human fetal and adult B-lymphopoiesis differ significantly, very few studies have directly compared the molecular pathways underlying these differences. However, both human adult (35, 41, 83) and fetal (1, 9, 84) RNA-seq data sets across many hematopoietic subpopulations have been produced separately and are publicly available for such analyses.

The advent of single cell sequencing technology has allowed the transcriptome of hematopoietic cells to be investigated in unprecedented detail. Recent single-cell transcriptome profiling of human FL and FBM hematopoiesis has demonstrated the transcriptomic changes that drive differentiation in the fetal B cell hierarchy from HSC to mature B-cells; with upregulation of genes such as *SPIB, SP100* and *CTSS* at HSC/MPP to B-lymphoid transition, followed by gradual upregulation of B-cell specific genes such as *MS4A1, CD79B*, and *DNTT* (1, 9).

Although fetal PreProB-progenitors are functionally identical to ProB-progenitors in being restricted to a B-lineage output; these two progenitor subtypes are molecularly distinct in their gene expression and chromatin accessibility patterns, with many myeloid (*MPO, CSF1R*), T-cell (*CD7, CD244*) and stem cell (*SPINK2, PROM1*) genes being accessible and expressed in PreProB-progenitors (1). In addition, when transcriptomes of iALL blasts are compared with different fetal HSPC populations, they most closely match the two fetal-specific progenitor populations, ELP and PreProB-progenitors (1) implicating these cells as potential targets for leukemic transformation.

Direct comparisons focusing on human B-progenitors showed that although adult and fetal counterparts were functionally similar, they did exhibit ontogeny-related transcriptomic differences at a single cell level, with fetal B-progenitors expressing high levels of genes involved in DNA recombination (*DNTT, RAG1*), as well as myeloid genes and known fetal-specific genes such as *LIN28B* (1, 80).

Previous studies have also shown that B cell receptor (BCR) development differs in fetal life, in particular with respect to V_H-D_H-J_H joining (85). Fetal BCR have a shorter CDR3 length, and show preferential usage of VH6, DHQ52 and the JH3 and JH4 loci compared to postnatal B-cells (60, 86–89).

RELEVANCE TO CHILDHOOD-ALL

The practical importance of characterizing human fetal B-lymphopoiesis is to understand the origins of childhood B-ALL, many of which are initiated before birth. This has led to the suggestion that fetal specific B1 B-cells and their progenitors could be the target cells for leukemia initiation in many subtypes of childhood leukemia. Gene expression signatures from mice which distinguish B1 and B2 B-cells have been mapped to human orthologs; application of these signatures to human pediatric ALL transcriptomic datasets separates B1 B-cell-like ALL subtypes including *ETV6-RUNX1* ALL, from B2 B-cell-like subtypes such as *BCR-ABL1*, hyperdiploid, and *KMT2A* ALL subtypes (90). Intriguingly, in murine models BCR-ABL transduction into B1 B-progenitors yields greater tumor burden in resulting murine leukemia than B2 B-progenitors (91).

These data suggest that it is likely that the biology of different types of infant/childhood Precursor B-ALL depends on the developmental stage specific characteristics of the leukemia-initiating cell although this remains to be demonstrated directly. Nevertheless, it is likely that this is particularly relevant for iALL, which invariably originates *in utero* and presents as a rapid onset aggressive leukemia within the 1st year of life.

Clinical and Biological Features of Infant and Childhood-ALL

The clinical course and molecular features of iALL are distinct from childhood-ALL. iALL remains a disease with dismal event-free survival (EFS) (18, 92–94), although recent risk-stratified treatment protocols suggest that outcomes could be improved (95). In iALL, blasts are predominantly $CD19^{+}CD10^{-}$, often with aberrant myeloid cell surface markers suggestive of an immature B-progenitor, as opposed to a $CD19^{+}CD10^{+}$ Pre-B phenotype in childhood-ALL (18, 96). *KMT2A* gene

rearrangements (*KMT2A-r*) is the main genetic driver for 70–80% iALL cases, as opposed to only 2–5% of childhood-ALL cases (97, 98).

Current evidence suggests that iALL (particularly *KMT2A-r* ALL) originates *in utero* and has been traced back to its fetal origin through retrospective detection of the fusion gene in neonatal blood spots (99), as well as studies in monozygotic twins with ALL (100, 101). A characteristic feature of iALL is the fact that a single hit (*KMT2A-r*) before birth seems to be sufficient to induce a rapidly-proliferating, therapy-resistant leukemia without the need for additional mutations (102).

Unlike iALL, many cases of childhood-ALL also originate *in utero* but only develop into full-blown leukemia after a second post-natal hit (15, 16). Several subtypes of childhood B-ALL have been shown to arise *in utero* including those characterized by *KMT2A-r* (103, 104), *ETV6-RUNX1* (105–107), *BCR-ABL* (108), *TCF3-PBX1* (109), *TCF3-ZNF384* (110) gene fusions and high hyperdiploid ALL (111, 112) (**Figure 2**).

There are several properties of fetal hematopoietic cells that may underlie the pathogenesis of iALL and childhood-ALL. Firstly, fetal HSPC are more proliferative (6, 7) and have better long term repopulating ability in xenograft models (8, 113–115). Fetal-specific gene expression programs such as the *LIN28B-LET-7-HMGA2* axis (79, 80, 116, 117) have been shown to drive self-renewal (118) and oncogenesis (10–12, 119). Activation of *LIN28B*, in particular, has been demonstrated in several cancers and results in suppression of *LET-7* micro-RNAs and subsequent de-repression of an array of oncogenes including *MYC, RAS, BLIMP1, ARID3A* and *HMGA2* (10, 120). *ARID3A* is necessary for fetal B lymphopoiesis and B1 cell division (121, 122), and has also been shown to promote cancers by driving higher *MYC* expression (123, 124). *HMGA2* is a fetal-specific transcription factor that is re-expressed in many cancers. It promotes cell proliferation, and the Lin28-Let-7-HMGA2 axis maintains cancers in an undifferentiated state (125). The expression of oncogenes such as *LIN28B* in fetal HSPC, may therefore play a role in leukemia initiation and transformation of fetal target cells, and in particular the development of aggressive leukemias in infancy and early childhood.

Secondly, there is a higher proportion of B-progenitors in fetal life compared to adults (1, 2). B-lymphopoiesis itself changes through the human lifetime with a switch in the ratio of B-progenitors to more mature B-cells (30, 49). Regardless of the mechanism, hematopoiesis in the human FBM is skewed toward the B-lymphoid lineage with the presence of a very high frequency of B-progenitors (1, 126) thus expanding the pool of target cells for malignant transformation.

Developmental Origins of iALL

It is also possible that the fetal cell of origin for iALL and childhood-ALL are different (**Figure 2**). We suggest that an attractive hypothesis is that iALL arises in a unique B-progenitor found only in fetal life. Of particular interest are fetal-specific IL7R$^+$ ELP (1, 50, 53, 54) and PreProB-progenitors (1, 50, 55, 56) that share immunophenotypic, transcriptomic and *IgH* rearrangement patterns with iALL blasts (1, 96). Compared to ABM counterparts, fetal PreProB-progenitors uniquely express known oncofetal genes, such as *LIN28B*, as well as genes implicated in *KMT2A-r* iALL, such as *KLRK1* and *PPP1R14A* (127, 128) that have not previously been recognized as being fetal-specific (1). Fetal ELP/PreProB-progenitors also demonstrate features that could account for lineage plasticity such as an accessible chromatin pattern, together with residual expression, of myeloid and stem cell genes (1). In addition, iALL can switch to a myeloid lineage at relapse, especially after B-lymphoid directed treatment (129–132). This could either be a feature of residual preleukemic primitive progenitors that are capable of giving rise to both myeloid and lymphoid leukemia, or because of plasticity and/or reprogramming of leukemic early B-lymphoid progenitors (130, 133). For example, *KMT2A-r*, the most frequent genetic driver of iALL, may drive leukemogenesis by binding to accessible genes in permissive fetal progenitors; or indeed alter the chromatin accessibility and gene expression patterns of target genes. *KMT2A* is a lysine methyltransferase, and *KMT2A-r* is thought to promote leukemogenesis by activating key target genes such as *HOXA9* and *MEIS1* (134, 135). Although there is some heterogeneity in *KMT2A-r* ALL based on the specific fusion partner gene, most KMT2A-fusion proteins drive and maintain leukemia via a protein complex involving AF4/ENL/AF9/PTEF-B. KMT2A-fusion proteins bind directly to gene targets where they aberrantly upregulate gene expression, partly by increasing histone-3-lysine-79 dimethylation through DOT1L (135).

These mechanisms of *KMT2A-r* mediated transformation are difficult to validate without a *bona fide* model of iALL, which has been very difficult to generate. However, we have recently developed a novel iALL model derived by CRISPR-Cas9 mediated *KMT2A-r* in primary human FL HSPC (136). This demonstrates that a human fetal cell context is permissive, and indeed probably required; to give rise to an ALL that recapitulates key features of iALL. In this model, recruitment of fetal-specific genes by KMT2A-AF4 is demonstrated by KMT2A-N and AF4-C binding and H3K79me2 at these genes by ChIP-seq (136). Furthermore, maintenance of fetal-specific gene expression programs accounts for the unique molecular profile of iALL, suggesting that it is the specific fetal target cell(s) in which it arises that provide the permissive cellular context (136).

Developmental Origins of Childhood-ALL

It is possible that childhood-ALL on the other hand is likely to arise from a more mature CD19$^+$CD10$^+$ fetal B-progenitor such as ProB-progenitors or PreB-cells. These cell populations are found in abundance in FBM and expand rapidly throughout the second trimester. As in iALL, several genes that have been implicated in the pathogenesis of childhood-ALL are also important in fetal B lymphoid development. Some of these, such as *PAX5, EBF1, TCF3,* and *IL7R* (137, 138), are expressed at higher levels in fetal B-progenitors compared to postnatal counterparts (1). This is also true for the B-cell specific gene *RAG1* that may play a role in driving childhood-ALL-associated chromosomal translocations such as *ETV6-RUNX1* (139). In addition, childhood-ALL is characterized by multiple lesions affecting cell cycle and B-cell differentiation genes (138). It is hypothesized that the proliferative capacity and

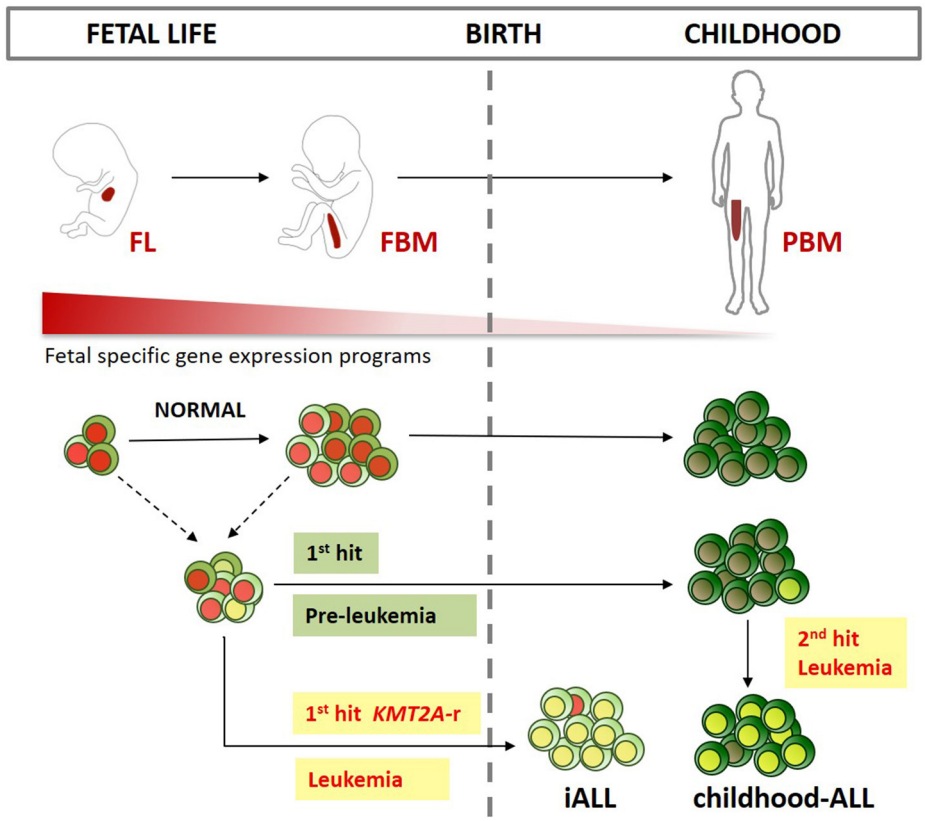

FIGURE 2 | Developmental origins of ALL. Schematic representation of the main sites of early life B-lymphopoiesis (FL, fetal liver; FBM, fetal bone marrow; PBM, pediatric BM). Different fetal lymphoid progenitors could be the target cell for infant ALL (iALL), and childhood-ALL. While iALL can develop after just one intrauterine "hit" such as *KMT2A* gene rearrangement (*KMT2A-r*), childhood-ALL usually develops after a second postnatal hit. Fetal-specific gene expression programs are down regulated after birth. These programs might be key in providing a permissive cellular context for prenatal B-progenitor leukemia initiation in specific target cells as described above.

complementary epigenetic profile (such as greater chromatin accessibility of highly expressed genes) of the cell of origin provide the right substrate for leukemic transformation (35, 140). This permissive cell-state is likely to be present in FBM ProB-progenitors where their rapid proliferation at the expense of differentiation during a particular developmental time window may make them more susceptible to oncogenic hits. Others have hypothesized that it is the fetal/neonatal BM niche that drives the lymphoid-biased phenotype of *KMT2A-r* infant/childhood leukemia (141).

CONCLUSION

Recent advances in developmental hematopoiesis have allowed better characterization of human fetal B-lymphopoiesis using molecular and functional studies. This has revealed fetal-specific B-lymphoid progenitors and B-cell developmental pathways that can be distinguished from postnatal B-lymphopoiesis. Lineage specification of fetal progenitors, the enrichment of multi/oligopotent progenitors and their proliferative capacity is also likely to be driven by microenvironmental cues from the FL and FBM hematopoietic niche.

Studies directly comparing fetal B-lymphoid cells and their microenvironment with childhood and adult counterparts are crucial if we are to understand the site- and stage-specific variation in hematopoiesis throughout the human lifetime and the role it plays in normal and abnormal B-lymphopoiesis. This also has implications for using age-appropriate controls for studies of disorders of hematopoiesis, particularly in early life.

The lymphoid bias of normal fetal hematopoiesis may well be a key factor in the predominance of ALL among infants and children. A better understanding of the importance of the fetal context for leukemogenesis is likely to require models derived from human fetal HSPCs and/or niche. Using human fetal cells to develop faithful infant and childhood-ALL models will allow better understanding of disease pathogenesis and rational development and testing of therapeutics in the future.

AUTHOR CONTRIBUTIONS

TJ, RL, and AR drafted the manuscript. AR reviewed and edited the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. O'Byrne S, Elliott N, Rice S, Buck G, Fordham N, Garnett C, et al. Discovery of a CD10-negative B-progenitor in human fetal life identifies unique ontogeny-related developmental programs. *Blood*. (2019) 134:1059–71. doi: 10.1182/blood.2019001289
2. Rossi MI, Yokota T, Medina KL, Garrett KP, Comp PC, Schipul AH Jr, et al. B lymphopoiesis is active throughout human life, but there are developmental age-related changes. *Blood*. (2003) 101:576–84. doi: 10.1182/blood-2002-03-0896
3. Pang WW, Price EA, Sahoo D, Beerman I, Maloney WJ, Rossi DJ, et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc Natl Acad Sci*. (2011) 108:20012. doi: 10.1073/pnas.1116110108
4. Rundberg Nilsson A, Soneji S, Adolfsson S, Bryder D, Pronk CJ. Human and murine hematopoietic stem cell aging is associated with functional impairments and intrinsic megakaryocytic/erythroid bias. *PLoS ONE*. (2016) 11:e0158369. doi: 10.1371/journal.pone.0158369
5. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science*. (2016) 351:aab2116-aab. doi: 10.1126/science.aab2116
6. Lansdorp PM, Dragowska W, Mayani H. Ontogeny-related changes in proliferative potential of human hematopoietic cells. *J Exp Med*. (1993) 178:787–91. doi: 10.1084/jem.178.3.787
7. Muench MO, Cupp J, Polakoff J, Roncarolo MG. Expression of CD33, CD38, and HLA-DR on CD34+ human fetal liver progenitors with a high proliferative potential. *Blood*. (1994) 83:3170–81. doi: 10.1182/blood.V83.11.3170.bloodjournal83113170
8. Harrison DE, Zhong RK, Jordan CT, Lemischka IR, Astle CM. Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. *Exp Hematol*. (1997) 25:293–7.
9. Popescu D-M, Botting RA, Stephenson E, Green K, Webb S, Jardine L, et al. Decoding human fetal liver haematopoiesis. *Nature*. (2019) 574:365–71. doi: 10.1038/s41586-019-1652-y
10. Zhou J, Ng S-B, Chng W-J. LIN28/LIN28B: an emerging oncogenic driver in cancer stem cells. *Int J Biochem Cell Biol*. (2013) 45:973–8. doi: 10.1016/j.biocel.2013.02.006
11. Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, et al. Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet*. (2009) 41:843–8. doi: 10.1038/ng.392
12. Elcheva IA, Wood T, Chiarolanzio K, Chim B, Wong M, Singh V, et al. RNA-binding protein IGF2BP1 maintains leukemia stem cell properties by regulating HOXB4, MYB, and ALDH1A1. *Leukemia*. (2020) 34:1354–63. doi: 10.1038/s41375-019-0656-9
13. Kuijk E, Blokzijl F, Jager M, Besselink N, Boymans S, Chuva De Sousa Lopes SM, et al. Early divergence of mutational processes in human fetal tissues. *Sci Adv*. (2019) 5:eaaw1271. doi: 10.1126/sciadv.aaw1271
14. Hasaart KAL, Manders F, van der Hoorn M-L, Verheul M, Poplonski T, Kuijk E, et al. Mutation accumulation and developmental lineages in normal and Down syndrome human fetal haematopoiesis. *Sci Rep*. (2020) 10:12991. doi: 10.1038/s41598-020-69822-1
15. Greaves M. *In utero* origins of childhood leukaemia. *Early Hum Dev*. (2005) 81:123–9. doi: 10.1016/j.earlhumdev.2004.10.004
16. Greaves M. A causal mechanism for childhood acute lymphoblastic leukaemia. *Nat Rev Cancer*. (2018) 18:471–84. doi: 10.1038/s41568-018-0015-6
17. Vora A, Goulden N, Wade R, Mitchell C, Hancock J, Hough R, et al. Treatment reduction for children and young adults with low-risk acute lymphoblastic leukaemia defined by minimal residual disease (UKALL 2003): a randomised controlled trial. *Lancet Oncol*. (2013) 14:199–209. doi: 10.1016/S1470-2045(12)70600-9
18. Pieters R, De Lorenzo P, Ancliffe P, Aversa LA, Brethon B, Biondi A, et al. Outcome of infants younger than 1 year with acute lymphoblastic leukemia treated with the interfant-06 protocol: results from an international phase III randomized study. *J Clin Oncol*. (2019) 37:2246. doi: 10.1200/JCO.19.00261
19. Doulatov S, Notta F, Laurenti E, John. Hematopoiesis: a human perspective. *Cell Stem Cell*. (2012) 10:120–36. doi: 10.1016/j.stem.2012.01.006
20. Ernst PB, Carvunis AR. Of mice, men and immunity: a case for evolutionary systems biology. *Nat Immunol*. (2018) 19:421–5. doi: 10.1038/s41590-018-0084-4
21. Milne TA. Mouse models of MLL leukemia: recapitulating the human disease. *Blood*. (2017) 129:2217–23. doi: 10.1182/blood-2016-10-691428
22. Rice S, Roy A. MLL-rearranged infant leukaemia: a 'thorn in the side' of a remarkable success story. *Biochim Biophys Acta Gene Regul Mech*. (2020) 1863:194564. doi: 10.1016/j.bbagrm.2020.194564
23. Höfer T, Rodewald H-R. Differentiation-based model of hematopoietic stem cell functions and lineage pathways. *Blood*. (2018) 132:1106–13. doi: 10.1182/blood-2018-03-791517
24. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood*. (2015) 125:2605–13. doi: 10.1182/blood-2014-12-570200
25. Kohn LA, Hao Q-L, Sasidharan R, Parekh C, Ge S, Zhu Y, et al. Lymphoid priming in human bone marrow begins before expression of CD10 with upregulation of L-selectin. *Nat Immunol*. (2012) 13:963–71. doi: 10.1038/ni.2405
26. Luc S, Buza-Vidas N, Jacobsen SEW. Biological and molecular evidence for existence of lymphoid-primed multipotent progenitors. *Ann N Y Acad Sci*. (2007) 1106:89–94. doi: 10.1196/annals.1392.023
27. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol*. (2010) 11:585–93. doi: 10.1038/ni.1889
28. Karamitros D, Stoilova B, Aboukhalil Z, Hamey F, Reinisch A, Samitsch M, et al. Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells. *Nat Immunol*. (2018) 19:85–97. doi: 10.1038/s41590-017-0001-2
29. Galy A, Travis M, Cen D, Chen B. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity*. (1995) 3:459–73. doi: 10.1016/1074-7613(95)90175-2
30. Nuñez C, Nishimoto N, Gartland GL, Billips LG, Burrows PD, Kubagawa H, et al. B cells are generated throughout life in humans. *J Immunol*. (1996) 156:866–72.
31. LeBien TW. Fates of human B-cell precursors. *Blood*. (2000) 96:9–23. doi: 10.1182/blood.V96.1.9.013k27_9_23
32. Hystad ME, Myklebust JH, Bo TH, Sivertsen EA, Rian E, Forfang L, et al. Characterization of early stages of human B cell development by gene expression profiling. *J Immunol*. (2007) 179:3662–71. doi: 10.4049/jimmunol.179.6.3662

33. Boller S, Grosschedl R. The regulatory network of B-cell differentiation: a focused view of early B-cell factor 1 function. *Immunol Rev*. (2014) 261:102–15. doi: 10.1111/imr.12206
34. Blom B, Spits H. Development of human lymphoid cells. *Annu Rev Immunol*. (2006) 24:287–320. doi: 10.1146/annurev.immunol.24.021605.090612
35. Corces MR, Buenrostro JD, Wu B, Greenside PG, Chan SM, Koenig JL, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet*. (2016) 48:1193–203. doi: 10.1038/ng.3646
36. Van Zelm MC, Van Der Burg M, De Ridder D, Barendregt BH, De Haas EFE, Reinders MJT, et al. Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression. *J Immunol*. (2005) 175:5912–22. doi: 10.4049/jimmunol.175.9.5912
37. Laurenti E, Göttgens B. From haematopoietic stem cells to complex differentiation landscapes. *Nature*. (2018) 553:418–26. doi: 10.1038/nature25022
38. Velten L, Haas SF, Raffel S, Blaszkiewicz S, Islam S, Hennig BP, et al. Human haematopoietic stem cell lineage commitment is a continuous process. *Nat Cell Biol*. (2017) 19:271–81. doi: 10.1038/ncb3493
39. Zheng S, Papalexi E, Butler A, Stephenson W, Satija R. Molecular transitions in early progenitors during human cord blood hematopoiesis. *Mol Syst Biol*. (2018) 14:e8041-e. doi: 10.15252/msb.20178041
40. Bendall SC, Davis KL, Amir el AD, Tadmor MD, Simonds EF, Chen TJ, et al. Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. *Cell*. (2014) 157:714–25. doi: 10.1016/j.cell.2014.04.005
41. Hay SB, Ferchen K, Chetal K, Grimes HL, Salomonis N. The Human Cell Atlas bone marrow single-cell interactive web portal. *Exp Hematol*. (2018) 68:51–61. doi: 10.1016/j.exphem.2018.09.004
42. Hardy RR, Hayakawa K. Perspectives on fetal derived CD5+B1 B cells. *Eur J Immunol*. (2015) 45:2978–84. doi: 10.1002/eji.201445146
43. Tavian M, Hallais MF, Peault B. Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development*. (1999) 126:793–803.
44. Tavian M, Coulombel L, Luton D, Clemente HS, Dieterlen-Lievre F, Peault B. Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood*. (1996) 87:67–72. doi: 10.1182/blood.V87.1.67.67
45. Tavian M, Peault B. Embryonic development of the human hematopoietic system. *Int J Dev Biol*. (2005) 49:243–50. doi: 10.1387/ijdb.041957mt
46. Copley MR, Eaves CJ. Developmental changes in hematopoietic stem cell properties. *Exp Mol Med*. (2013) 45:e55. doi: 10.1038/emm.2013.98
47. Ivanovs A, Rybtsov S, Ng ES, Stanley EG, Elefanty AG, Medvinsky A. Human haematopoietic stem cell development: from the embryo to the dish. *Development*. (2017) 144:2323–37. doi: 10.1242/dev.134866
48. Charbord P, Tavian M, Humeau L, Peault B. Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood*. (1996) 87:4109–19. doi: 10.1182/blood.V87.10.4109.bloodjournal87104109
49. Asma GE, Langlois van den Bergh R, Vossen JM. Development of pre-B and B lymphocytes in the human fetus. *Clin Exp Immunol*. (1984) 56:407–14.
50. Roy A, Cowan G, Mead AJ, Filippi S, Bohn G, Chaidos A, et al. Perturbation of fetal liver hematopoietic stem and progenitor cell development by trisomy 21. *Proc Natl Acad Sci*. (2012) 109:17579–84. doi: 10.1073/pnas.12114 05109
51. Tavian M, Biasch K, Sinka L, Vallet J, Peault B. Embryonic origin of human hematopoiesis. *Int J Dev Biol*. (2010) 54:1061–5. doi: 10.1387/ijdb.103097mt
52. Böiers C, Carrelha J, Lutteropp M, Luc S, Joanna, Azzoni E, et al. Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell*. (2013) 13:535–48. doi: 10.1016/j.stem.2013.08.012
53. Alhaj Hussen K, Vu Manh TP, Guimiot F, Nelson E, Chabaane E, Delord M, et al. Molecular and functional characterization of lymphoid progenitor subsets reveals a bipartite architecture of human lymphopoiesis. *Immunity*. (2017) 47:680–96 e8. doi: 10.1016/j.immuni.2017.09.009
54. Böiers C, Richardson SE, Laycock E, Zriwil A, Turati VA, Brown J, et al. A human IPS model implicates embryonic B-myeloid fate restriction as developmental susceptibility to B Acute lymphoblastic leukemia-associated ETV6-RUNX1. *Dev Cell*. (2018) 44:362–77.e7. doi: 10.1016/j.devcel.2017.12.005
55. Sanz E, Alvarez-Mon M, Martinez AC, de la Hera A. Human cord blood CD34+Pax-5+ B-cell progenitors: single-cell analyses of their gene expression profiles. *Blood*. (2003) 101:3424–30. doi: 10.1182/blood-2002-07-2244
56. Sanz E, Munoz AN, Monserrat J, Van-Den-Rym A, Escoll P, Ranz I, et al. Ordering human CD34+CD10-CD19+ pre/pro-B-cell and CD19- common lymphoid progenitor stages in two pro-B-cell development pathways. *Proc Natl Acad Sci U S A*. (2010) 107:5925–30. doi: 10.1073/pnas.0907942107
57. Grumayer ER, Griesinger F, Hummell DS, Brunning RD, Kersey JH. Identification of novel B-lineage cells in human fetal bone marrow that coexpress CD7. *Blood*. (1991) 77:64–8. doi: 10.1182/blood.V77.1.64.64
58. Uckun FM, Ledbetter JA. Immunobiologic differences between normal and leukemic human B-cell precursors. *Proc Natl Acad Sci U S A*. (1988) 85:8603–7. doi: 10.1073/pnas.85.22.8603
59. Bueno C, Van Roon EHJ, Muñoz-López A, Sanjuan-Pla A, Juan M, Navarro A, et al. Immunophenotypic analysis and quantification of B-1 and B-2 B cells during human fetal hematopoietic development. *Leukemia*. (2016) 30:1603–6. doi: 10.1038/leu.2015.362
60. Roy A, Bystry V, Bohn G, Goudevenou K, Reigl T, Papaioannou M, et al. High resolution IgH repertoire analysis reveals fetal liver as the likely origin of life-long, innate B lymphopoiesis in humans. *Clin Immunol*. (2017) 183:8–16. doi: 10.1016/j.clim.2017.06.005
61. Rechavi E, Lev A, Lee YN, Simon AJ, Yinon Y, Lipitz S, et al. Timely and spatially regulated maturation of B and T cell repertoire during human fetal development. *Sci Transl Med*. (2015) 7:276ra25. doi: 10.1126/scitranslmed.aaa0072
62. Berman JE, Nickerson KG, Pollock RR, Barth JE, Schuurman RK, Knowles DM, et al. VH gene usage in humans: biased usage of the VH6 gene in immature B lymphoid cells. *Eur J Immunol*. (1991) 21:1311–4. doi: 10.1002/eji.1830210532
63. Pascual V, Verkruyse L, Casey ML, Capra JD. Analysis of Ig H chain gene segment utilization in human fetal liver. Revisiting the "proximal utilization hypothesis". *J Immunol*. (1993) 151:4164–72.
64. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med*. (1983) 157:202–18. doi: 10.1084/jem.157.1.202
65. Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J Exp Med*. (1985) 161:1554–68. doi: 10.1084/jem.161.6.1554
66. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. *Nat Rev Cancer*. (2016) 16:145–62. doi: 10.1038/nrc.2016.8
67. Kantor AB, Herzenberg LA. Origin of murine B cell lineages. *Annu Rev Immunol*. (1993) 11:501–38. doi: 10.1146/annurev.iy.11.040193.002441
68. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Annu Rev Immunol*. (2011) 11:34–46. doi: 10.1038/nri2901
69. Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, Porayette P, Shelley WC, Conway SJ, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci U S A*. (2011) 108:1468–73. doi: 10.1073/pnas.1015841108
70. Kristiansen TA, Vanhee S, Yuan J. The influence of developmental timing on B cell diversity. *Curr Opin Immunol*. (2018) 51:7–13. doi: 10.1016/j.coi.2017.12.005
71. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell–specified progenitor. *Nature Immunology*. (2006) 7:293–301. doi: 10.1038/ni1301
72. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity*. (2012) 36:13–21. doi: 10.1016/j.immuni.2011.11.017
73. Lee J, Kuchen S, Fischer R, Chang S, Lipsky PE. Identification and characterization of a human CD5+ pre-naive B cell population. *J Immunol*. (2009) 182:4116–26. doi: 10.4049/jimmunol.0803391
74. Taniguchi O, Miyajima H, Hirano T, Noguchi M, Ueda A, Hashimoto H, et al. The Leu-1 B-cell subpopulation in patients with rheumatoid arthritis. *J Clin Immunol*. (1987) 7:441–8. doi: 10.1007/BF00915053

75. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+CD27+CD43+CD70–. *J Exp Med*. (2011) 208:67–80. doi: 10.1084/jem.20101499
76. Rodriguez-Zhurbenko N, Quach TD, Hopkins TJ, Rothstein TL, Hernandez AM. Human B-1 cells and B-1 cell antibodies change with advancing age. *Front Immunol*. (2019) 10:483. doi: 10.3389/fimmu.2019.00483
77. Descatoire M, Weill J-C, Reynaud C-A, Weller S. A human equivalent of mouse B-1 cells? *J Exp Med*. (2011) 208:2563–4. doi: 10.1084/jem.20112232
78. Quách TD, Rodríguez-Zhurbenko N, Hopkins TJ, Guo X, Hernández AM, Li W, et al. Distinctions among circulating antibody-secreting cell populations, including B-1 cells, in human adult peripheral blood. *J Immunol*. (2016) 196:1060–9. doi: 10.4049/jimmunol.1501843
79. McWilliams L, Su K-Y, Liang X, Liao D, Floyd S, Amos J, et al. The human fetal lymphocyte lineage: identification by CD27 and LIN28B expression in B cell progenitors. *J Leukoc Biol*. (2013) 94:991–1001. doi: 10.1189/jlb.0113048
80. Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science*. (2012) 335:1195–200. doi: 10.1126/science.1216557
81. Xu X, Deobagkar-Lele M, Bull KR, Crockford TL, Mead AJ, Cribbs AP, et al. An ontogenetic switch drives the positive and negative selection of B cells. *Proc Natl Acad Sci*. (2020) 117:3718–27. doi: 10.1073/pnas.1915247117
82. Montecino-Rodriguez E, Fice M, Casero D, Berent-Maoz B, Barber CL, Dorshkind K. Distinct genetic networks orchestrate the emergence of specific waves of fetal and adult B-1 and B-2 development. *Immunity*. (2016) 45:527–39. doi: 10.1016/j.immuni.2016.07.012
83. Quek L, Otto GW, Garnett C, Lhermitte L, Karamitros D, Stoilova B, et al. Genetically distinct leukemic stem cells in human CD34- acute myeloid leukemia are arrested at a hemopoietic precursor-like stage. *J Exp Med*. (2016) 213:1513–35. doi: 10.1084/jem.20151775
84. Agraz-Doblas A, Bueno C, Bashford-Rogers R, Roy A, Schneider P, Bardini M, et al. Unravelling the cellular origin and clinical prognostic markers of infant B-cell acute lymphoblastic leukemia using genome-wide analysis. *Haematologica*. (2019) 104:1176–88. doi: 10.3324/haematol.2018.206375
85. Schroeder HW Jr, Mortari F, Shiokawa S, Kirkham PM, Elgavish RA, Bertrand FE III. Developmental regulation of the human antibody repertoire. *Ann N Y Acad Sci*. (1995) 764:242–60. doi: 10.1111/j.1749-6632.1995.tb55834.x
86. Schroeder HW Jr, Wang JY. Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. *Proc Natl Acad Sci U S A*. (1990) 87:6146–50. doi: 10.1073/pnas.87.16.6146
87. Souto-Carneiro MM, Sims GP, Girschik H, Lee J, Lipsky PE. Developmental changes in the human heavy chain CDR3. *J Immunol*. (2005) 175:7425–36. doi: 10.4049/jimmunol.175.11.7425
88. Van Es JH, Raaphorst FM, van Tol MJ, Meyling FH, Logtenberg T. Expression pattern of the most JH-proximal human VH gene segment (VH6) in the B cell and antibody repertoire suggests a role of VH6-encoded IgM antibodies in early ontogeny. *J Immunol*. (1993) 150:161–8.
89. Bertrand FE III, Billips LG, Burrows PD, Gartland GL, Kubagawa H, Schroeder HW Jr. Ig D(H) gene segment transcription and rearrangement before surface expression of the pan-B-cell marker CD19 in normal human bone marrow. *Blood*. (1997) 90:736–44. doi: 10.1182/blood.V90.2.736.736_736_744
90. Fitch B, Roy R, Geng H, Montecino-Rodriguez E, Bengtsson H, Gaillard C, et al. Human pediatric B-cell acute lymphoblastic leukemias can be classified as B-1 or B-2-like based on a minimal transcriptional signature. *Exp Hematol*. (2020) 90:65–71.e1. doi: 10.1016/j.exphem.2020.09.184
91. Montecino-Rodriguez E, Li K, Fice M, Dorshkind K. Murine B-1 B cell progenitors initiate B-acute lymphoblastic leukemia with features of high-risk disease. *J Immunol*. (2014) 192:5171–8. doi: 10.4049/jimmunol.1303170
92. Dreyer ZE, Hilden JM, Jones TL, Devidas M, Winick NJ, Willman CL, et al. Intensified chemotherapy without SCT in infant ALL: results from COG P9407 (Cohort 3). *Pediatr Blood Cancer*. (2015) 62:419–26. doi: 10.1002/pbc.25322
93. Hilden JM, Dinndorf PA, Meerbaum SO, Sather H, Villaluna D, Heerema NA, et al. Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. *Blood*. (2006) 108:441–51. doi: 10.1182/blood-2005-07-3011
94. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. (2007) 370:240–50. doi: 10.1016/S0140-6736(07)61126-X
95. Tomizawa D, Miyamura T, Imamura T, Watanabe T, Moriya Saito A, Ogawa A, et al. A risk-stratified therapy for infants with acute lymphoblastic leukemia: a report from the JPLSG MLL-10 trial. *Blood*. (2020) 136:1813–23. doi: 10.1182/blood.2019004741
96. Jansen MW, Corral L, van der Velden VH, Panzer-Grumayer R, Schrappe M, Schrauder A, et al. Immunobiological diversity in infant acute lymphoblastic leukemia is related to the occurrence and type of MLL gene rearrangement. *Leukemia*. (2007) 21:633–41. doi: 10.1038/sj.leu.2404578
97. Iacobucci I, Mullighan CG. Genetic basis of acute lymphoblastic leukemia. *J Clin Oncol*. (2017) 35:975–83. doi: 10.1200/JCO.2016.70.7836
98. Moorman AV, Ensor HM, Richards SM, Chilton L, Schwab C, Kinsey SE, et al. Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: results from the UK Medical Research Council ALL97/99 randomised trial. *Lancet Oncol*. (2010) 11:429–38. doi: 10.1016/S1470-2045(10)70066-8
99. Gale KB, Ford AM, Repp R, Borkhardt A, Keller C, Eden OB, et al. Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. *Proc Natl Acad Sci U S A*. (1997) 94:13950–4. doi: 10.1073/pnas.94.25.13950
100. Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. *Blood*. (2003) 102:2321–33. doi: 10.1182/blood-2002-12-3817
101. Greaves MF, Wiemels J. Origins of chromosome translocations in childhood leukaemia. *Nat Rev Cancer*. (2003) 3:639–49. doi: 10.1038/nrc1164
102. Andersson AK, Ma J, Wang J, Chen X, Gedman AL, Dang J, et al. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. *Nat Genet*. (2015) 47:330–7. doi: 10.1038/ng.3230
103. Ford AM, Ridge SA, Cabrera ME, Mahmoud H, Steel CM, Chan LC, et al. *In utero* rearrangements in the trithorax-related oncogene in infant leukaemias. *Nature*. (1993) 363:358–60. doi: 10.1038/363358a0
104. Jones LK, Neat MJ, van Delft FW, Mitchell MP, Adamaki M, Stoneham SJ, et al. Cryptic rearrangement involving MLL and AF10 occurring *in utero*. *Leukemia*. (2003) 17:1667–9. doi: 10.1038/sj.leu.2403039
105. Wiemels JL, Ford AM, Van Wering ER, Postma A, Greaves M. Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion *in utero*. *Blood*. (1999) 94:1057–62. doi: 10.1182/blood.V94.3.1057.415k10_1057_1062
106. Ford AM, Bennett CA, Price CM, Bruin MC, Van Wering ER, Greaves M. Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. *Proc Natl Acad Sci U S A*. (1998) 95:4584–8. doi: 10.1073/pnas.95.8.4584
107. Hong D, Gupta R, Ancliff P, Atzberger A, Brown J, Soneji S, et al. Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. *Science*. (2008) 319:336–9. doi: 10.1126/science.1150648
108. Cazzaniga G, van Delft FW, Lo Nigro L, Ford AM, Score J, Iacobucci I, et al. Developmental origins and impact of BCR-ABL1 fusion and IKZF1 deletions in monozygotic twins with Ph+ acute lymphoblastic leukemia. *Blood*. (2011) 118:5559–64. doi: 10.1182/blood-2011-07-366542
109. Hein D, Dreisig K, Metzler M, Izraeli S, Schmiegelow K, Borkhardt A, et al. The preleukemic TCF3-PBX1 gene fusion can be generated in utero and is present in ≈0.6% of healthy newborns. *Blood*. (2019) 134:1355–8. doi: 10.1182/blood.2019002215
110. Bueno C, Tejedor JR, Bashford-Rogers R, González-Silva L, Valdés-Mas R, Agraz-Doblás A, et al. Natural history and cell of origin of TCF3-ZNF384 and PTPN11 mutations in monozygotic twins with concordant BCP-ALL. *Blood*. (2019) 134:900–5. doi: 10.1182/blood.2019000893
111. Panzer-Grümayer ER, Fasching K, Panzer S, Hettinger K, Schmitt K, Stöckler-Ipsiroglu S, et al. Nondisjunction of chromosomes leading to hyperdiploid childhood B-cell precursor acute lymphoblastic leukemia is an early event during leukemogenesis. *Blood*. (2002) 100:347–9. doi: 10.1182/blood-2002-01-0144
112. Wiemels JL, Kang M, Chang JS, Zheng L, Kouyoumji C, Zhang L, et al. Backtracking RAS mutations in high hyperdiploid childhood

acute lymphoblastic leukemia. *Blood Cells Mol Dis*. (2010) 45:186–91. doi: 10.1016/j.bcmd.2010.07.007
113. Nicolini FE, Holyoake TL, Cashman JD, Chu PP, Lambie K, Eaves CJ. Unique differentiation programs of human fetal liver stem cells shown both *in vitro* and *in vivo* in NOD/SCID mice. *Blood*. (1999) 94:2686–95. doi: 10.1182/blood.V94.8.2686.420k15_2686_2695
114. Holyoake TL, Nicolini FE, Eaves CJ. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Exp Hematol*. (1999) 27:1418–27. doi: 10.1016/S0301-472X(99)00078-8
115. Beyer AI, Muench MO. Comparison of human hematopoietic reconstitution in different strains of immunodeficient mice. *Stem Cells Dev*. (2017) 26:102–12. doi: 10.1089/scd.2016.0083
116. Li YS, Zhou Y, Tang L, Shinton SA, Hayakawa K, Hardy RR. A developmental switch between fetal and adult B lymphopoiesis. *Ann N Y Acad Sci*. (2015) 1362:8–15. doi: 10.1111/nyas.12769
117. Zhou J, Bi C, Ching YQ, Chooi JY, Lu X, Quah JY, et al. Inhibition of LIN28B impairs leukemia cell growth and metabolism in acute myeloid leukemia. *J Hematol Oncol*. (2017) 10:138. doi: 10.1186/s13045-017-0507-y
118. Copley MR, Babovic S, Benz C, Knapp DJ, Beer PA, Kent DG, et al. The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. *Nat Cell Biol*. (2013) 15:916–25. doi: 10.1038/ncb2783
119. Oliveira-Mateos C, Sanchez-Castillo A, Soler M, Obiols-Guardia A, Pineyro D, Boque-Sastre R, et al. The transcribed pseudogene RPSAP52 enhances the oncofetal HMGA2-IGF2BP2-RAS axis through LIN28B-dependent and independent let-7 inhibition. *Nat Commun*. (2019) 10:3979. doi: 10.1038/s41467-019-11910-6
120. Balzeau J, Menezes MR, Cao S, Hagan JP. The LIN28/let-7 pathway in cancer. *Front Genet*. (2017) 8:31. doi: 10.3389/fgene.2017.00031
121. Hayakawa K, Li YS, Shinton SA, Bandi SR, Formica AM, Brill-Dashoff J, et al. Crucial role of increased Arid3a at the Pre-B and immature B cell stages for B1a cell generation. *Front Immunol*. (2019) 10:457. doi: 10.3389/fimmu.2019.00457
122. Zhou Y, Li YS, Bandi SR, Tang L, Shinton SA, Hayakawa K, et al. Lin28b promotes fetal B lymphopoiesis through the transcription factor Arid3a. *J Exp Med*. (2015) 212:569–80. doi: 10.1084/jem.20141510
123. Dausinas P, Pulakanti K, Rao S, Cole JM, Dahl R, Cowden Dahl KD. ARID3A and ARID3B induce stem promoting pathways in ovarian cancer cells. *Gene*. (2020) 738:144458. doi: 10.1016/j.gene.2020.144458
124. Tang J, Yang L, Li Y, Ning X, Chaulagain A, Wang T, et al. ARID3A promotes the development of colorectal cancer by upregulating AURKA. Carcinogenesis. (2020):bgaa118. doi: 10.1093/carcin/bgaa118. [Epub ahead of print].
125. Zhang S, Mo Q, Wang X. Oncological role of HMGA2 (Review). *Int J Oncol*. (2019) 55:775–88. doi: 10.3892/ijo.2019.4856
126. Roy A PBea. Single-cell profiling reveals key differences in the cellular architecture of human haematopoietic stem and progenitor cells throughout fetal and adult life (23rd Congress of the European Hematology Association Stockholm, Sweden, June 14-17, 2018). *HemaSphere*. (2018) 2:1–1113. doi: 10.1097/HS9.0000000000000060
127. Trentin L, Giordan M, Dingermann T, Basso G, Te Kronnie G, Marschalek R. Two independent gene signatures in pediatric t(4;11) acute lymphoblastic leukemia patients. *Eur J Haematol*. (2009) 83:406–19. doi: 10.1111/j.1600-0609.2009.01305.x
128. Armstrong SA, Staunton JE, Silverman LB, Pieters R, Den Boer ML, Minden MD, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*. (2002) 30:41–7. doi: 10.1038/ng765
129. Gardner R, Wu D, Cherian S, Fang M, Hanafi LA, Finney O, et al. Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy. *Blood*. (2016) 127:2406–10. doi: 10.1182/blood-2015-08-665547
130. Jacoby E, Nguyen SM, Fountaine TJ, Welp K, Gryder B, Qin H, et al. CD19 CAR immune pressure induces B-precursor acute lymphoblastic leukaemia lineage switch exposing inherent leukaemic plasticity. *Nat Commun*. (2016) 7:12320. doi: 10.1038/ncomms12320
131. Rayes A, McMasters RL, O'Brien MM. Lineage switch in MLL-rearranged infant leukemia following CD19-directed therapy. *Pediatr Blood Cancer*. (2016) 63:1113–5. doi: 10.1002/pbc.25953
132. Rossi JG, Bernasconi AR, Alonso CN, Rubio PL, Gallego MS, Carrara CA, et al. Lineage switch in childhood acute leukemia: an unusual event with poor outcome. *Am J Hematol*. (2012) 87:890–7. doi: 10.1002/ajh.23266
133. Wolfl M, Rasche M, Eyrich M, Schmid R, Reinhardt D, Schlegel PG. Spontaneous reversion of a lineage switch following an initial blinatumomab-induced ALL-to-AML switch in MLL-rearranged infant ALL. *Blood Adv*. (2018) 2:1382–5. doi: 10.1182/bloodadvances.2018018093
134. Ballabio E, Milne TA. Molecular and epigenetic mechanisms of MLL in human leukemogenesis. *Cancers*. (2012) 4:904–44. doi: 10.3390/cancers4030904
135. Takahashi S, Yokoyama A. The molecular functions of common and atypical MLL fusion protein complexes. *Biochim Biophys Acta Gene Regul Mech*. (2020) 1863:194548. doi: 10.1016/j.bbagrm.2020.194548
136. Rice S, Jackson T, Crump NT, Fordham N, Elliott N, O'Byrne S, et al. A novel human fetal liver-derived model reveals that MLL-AF4 drives a distinct fetal gene expression program in infant ALL. *bioRxiv*. (2020):2020.11.15.379990. doi: 10.1101/2020.11.15.379990. [Epub ahead of print].
137. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. (2007) 446:758–64. doi: 10.1038/nature05690
138. Kuiper RP, Schoenmakers EF, van Reijmersdal SV, Hehir-Kwa JY, van Kessel AG, van Leeuwen FN, et al. High-resolution genomic profiling of childhood ALL reveals novel recurrent genetic lesions affecting pathways involved in lymphocyte differentiation and cell cycle progression. *Leukemia*. (2007) 21:1258–66. doi: 10.1038/sj.leu.2404691
139. Papaemmanuil E, Rapado I, Li Y, Potter NE, Wedge DC, Tubio J, et al. RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia. *Nat Genet*. (2014) 46:116–25. doi: 10.1182/blood.V122.21.807.807
140. Shi Y, Su XB, He KY, Wu BH, Zhang BY, Han ZG. Chromatin accessibility contributes to simultaneous mutations of cancer genes. *Sci Rep*. (2016) 6:35270. doi: 10.1038/srep35270
141. Rowe RG, Lummertz da Rocha E, Sousa P, Missios P, Morse M, Marion W, et al. The developmental stage of the hematopoietic niche regulates lineage in MLL-rearranged leukemia. *J Exp Med*. (2019) 216:527–38. doi: 10.1084/jem.20181765

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TNFRSF13B Diversification Fueled by B Cell Responses to Environmental Challenges—A Hypothesis

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B cell differentiation and memory are controlled by the transmembrane activator and CAML interactor (TACI), a receptor encoded by *TNFRSF13B*. *TNFRSF13B* mutations are frequently found in common variable immunodeficiency (CVID) and in IgA -deficiency; yet, ∼98% of those with mutant *TNFRSF13B* are healthy. Indeed, *TNFRSF13B* is among the 5% most polymorphic genes in man. Other mammals evidence polymorphism at comparable loci. We hypothesize that *TNFRSF13B* diversity might promote rather than detract from well-being by controlling key elements of innate immunity. We shall discuss how extraordinary diversity of *TNFRSF13B* could have evolved and persisted across diverse species of mammals by controlling innate and adaptive B cell responses in apparently paradoxical ways.

Keywords: B-lymphocyte, *TNFRSF13B*, antibodies, T cell-dependent antibody response, T cell-independent antibody responses

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INTRODUCTION

B cell responses are often characterized as T cell-independent or T cell-dependent that differ on how T cells are engaged. In "T-independent B cell responses" antigens with repetitive epitopes, such as polysaccharides, engage B cell antigen receptors and/or toll-like receptors and in doing so induce proliferation and plasma cell differentiation. The antibodies produced in T cell-independent responses may appear relatively soon after introduction of antigen, but not immediately as in recall responses. The Ig variable region genes encoding these antibodies typically lack extensive somatic mutation (1). Repeated exposure to antigen that had generated a T cell-independent response does not hasten and amplify the response [i.e., B cell memory is not manifest; (1)]. In contrast, the T-dependent pathway requires T cell help and is associated with responses to protein antigens. Proteins are processed and peptides presented on major histocompatibility class II molecules expressed by antigen presenting cells that activate cognate CD4-positive T cells. These T cells in turn, engage cognate B cells activated by the same antigen by binding peptide MHC class-II complexes and co-receptors such as CD40. T cell-dependent responses induce long-lived memory responses and are associated with high affinity binding antibodies produced by plasma cells descending from the germinal centers in the secondary lymphoid organs (1). Naturally occurring polymorphisms in the *TNFRSF13B* gene differentially control T-dependent and T-independent pathways of antibody production.

TNFRSF13B AND THE CONTROL OF ANTIBODY PRODUCTION

TNFRSF13B encodes the tumor necrosis factor superfamily member 13B, a transmembrane receptor of lymphocytes that recognizes a proliferation induced ligand (APRIL) and B cell activation factor (BAFF), members of the tumor necrosis ligand family (2). TNFRSF13B also binds heparan sulfate chains associated with syndecan-2 and−4 cores (3). The signaling events initiated by TNFRSF13B are complex and intersect with signaling by Toll-Like receptors (TLRs) and will be only briefly summarized here. Binding of BAFF and APRIL to the cysteine rich domain of the receptor closest to the cell membrane (CRD2) engages TNFR—associated factors (TRAF 2, 5, and 6) and activates NF-kB, c-Jun NH2-terminal kinase (4) and activator protein 1 (AP-1) (5). TNFRSF13B interacts with calcium modulator and cyclophilin ligand (CAML), which in turn activates calcineurin and nuclear factor of activated T cells (NFAT) (6). TNFRSF13B is sometimes called "transmembrane activator and CAML interactor" or TACI (6) reflecting this series of interactions. TNFRSF13B potentiates signaling by Toll-like family receptors in B cells (7) and in macrophages (8). Accordingly, TNFRSF13B interacts with MyD88, recruits mechanistic target of rapamycin (mTOR), activates mTORC1 and NF-kB (9–11). TNFRSF13B signaling in B cells generates expression of BLIMP-1, a transcription factor that drives differentiation of B cells into long-lived plasma cells (12).

The importance of TNFRSF13B and BLIMP-1 for development of plasma cells and production of much of the Ig in blood was suggested by investigation of genetic basis of hypogammaglobulinemia, i.e., IgG-deficiency, IgM-deficiency, and IgA-deficiency observed in common variable immunodeficiency (CVID) and in selective IgA deficiency (13, 14). Consistent with this phenotype, TNFRSF13B-deficient mice have few plasma cells in secondary lymphoid organs and in the bone marrow and low concentrations of IgM, IgA, and IgG in serum (12). However, *TNFRSF13B* governs more than the machinery for long term-Ig production. Human subjects with CVID have an increased risk of lymphoma and gastro-intestinal cancer (15) and a propensity for development of autoimmunity (16). Mice with deficient tnfrsf13b exhibit pronounced expansion of follicular and germinal center B cells, despite hypogammaglobulinemia, suggesting tnfrsf13B may govern B cell differentiation and T and B cell interactions (12, 17, 18).

Although some functions of TNFRSF13B, such as control of plasma cell differentiation are understood, some puzzling contradictions remain. One contradiction concerns the impact of TNFRSF13B on the B cell response to antigen. TACI appears more or less essential for natural immunity because humans and mice lacking TACI (targeted deletion in mouse; expression of dominant-negative variants in humans) have extremely low levels of IgG, IgM, and IgA in blood (19) and produce little antigen specific antibodies after exposure to antigen or foreign organisms (20–22). However, most people with dominant negative TACI variants do not manifest immunodeficiency (23) and TACI knockout mice and mice expressing dominant negative TACI variants corresponding to those in humans mount proficient antibody responses and antibody-mediated defenses against pathogenic bacteria (17). Still more puzzling is the relationship between diversity of *TNFRSF13B* genotypes and phenotype. We shall describe recent work that may have begun to clarify apparently disparate aspects of the *TNFRSF13B* phenotype and identify yet unsettled questions we think of importance.

Recent investigations in mice and human subjects have clarified discrepancies concerning the impact of *TNFRSF13B* on B cell responses to antigen. It is now apparent that stimulation of TNFRSF13B is essential for T-independent- but not for T-cell-dependent B cell responses. The requirement for TNFRSF13B (TACI) function for mounting T-independent antibody responses was first shown by von Bulow et al. (24), who found that TACI-KO mice produce less antibodies in response to immunization with pneumococcus. Failure of T cell-independent responses in *tnfrsf13B*-mutant mice was confirmed by Wolf et al. (22) and Mantchev et al. (25), who showed the defective T-independent responses were due to a block in plasma cell differentiation. Tsuji et al. (12) found that defective plasma cell differentiation was due to defective Blimp-1 synthesis. Similarly, Grasset et al. (18) reported that production of gut IgA by T-independent response depends on *tnfrsf13B*. In contrast, human subjects with *TNFRSF13B* mutations, including those with dominant negative phenotype and mice with *tnfrsf13B* deficiency or dominant negative variants respond to antigen when T cell help is provided. Tsuji et al. (12, 17) reported that *tnfrsf13B*-deficient mice are quite proficient in responses to polypeptide antigens associated with enteric organisms (or purified therefrom), generating high affinity antibodies and conferring protection against reinfection. Likewise, Grasset et al. (18) found tnfrsf13B deficient mice quite effectively mount gut IgA responses if T cell help is present.

The differential impact of *TNFRSF13B* on T cell-independent and T-cell-dependent responses may reflect differences in how Blimp-1 is induced. In T cell-independent responses, induction of Blimp-1 depends absolutely on stimulation of TNFRSF13B by BAFF or APRIL (12, 17). In T cell-dependent responses, double strand DNA breaks generated by class-switch recombination (17) and/or engagement of CD40 and IL21/STAT3 (26) signaling can induce Blimp-1 independently of TNFRSF13B signaling. Although TNFRSF13B may not directly impact on Ig isotype class switching, changes of B cell development post-activation by TNFRSF13B mutation, or deletion, that interfere with signaling change the distribution of Ig isotypes in response to stimulation and in the steady state (27).

How TNFRSF13B signaling contributes to the generation and activation of memory B cells remains incompletely understood. The increase in TNFRSF13B expression by memory B cells suggests that TNFRSF13B signaling is important for memory B cell differentiation, survival or function (28). Indeed memory B cells from CVID subjects with monoallelic C104R or A181E mutations fail to activate in response to BCR, TLR7, or TLR9 stimuli (28). Memory B cells of subjects with TNFRSF13B hemizygosity (one null allele and one WT allele) mount a partial response to activation to BCR, TLR7, or TLR9 stimuli suggesting that signaling intensity is important in determining magnitude

of response (28). Whether or not the phenotypic manifestations of monoallelic dominant-negative mutations or haplo-deficiency impact memory B cell responses to T-dependent stimuli and in subjects who are healthy is not known.

TNFRSF13B POLYMORPHISMS

The diversity of *TNFRSF13B*, by some measures, equals or exceeds that of genes encoding the major histocompatibility complex (MHC) but the mechanisms contributing to *TNFRSF13B* diversity are less apparent. Diversity of MHC is generally ascribed to the function of housing a vast diversity of microbial peptides for presentation to T cells (29) and while some diversification likely reflects specificity for peptides, some may also reflect non-effector functions such as recognition leading to immune regulation for MHC-class II or controlling NK cells for MHC class I (29).

Yet, *TNFRSF13B* encodes a polypeptide the sole function of which may be recognition of several relatively non-polymorphic agonists, BAFF and APRIL (30). Why then do humans exhibit extreme genetic polymorphism of *TNFRSF13B?* There are 951 *TNFRSF13B* missense and only 383 synonymous mutations reported in compilations of more than 100.000 human genomes (https://useast.ensembl.org/index.html). Only 4 of the *TNFRSF13B* missense alleles are common (freq >0.05). Unlike *TNFRSF13B* variants, nearly 20% of HLA-A variants are found in 5% or more of the population (Genome Aggregation Database, v2.1.1; http://exac.broadinstitute.org). *TNFRSF13B* has a greater number of missense variants than it would be predicted by a sequence-context-based mutational model as reflected by a z score of −1.2, and *TNFRSF13B* has more observed than expected loss of function variants (LoF) (stop gain and splice site variants), which indicate a high tolerance to these types of mutations (pLI = 0.00, where pLI of 1 is the most intolerant) [Genome Aggregation Database, v2.1.1; http://exac.broadinstitute.org; (31)]. Most common variants are phenotypically dominant, either as dominant negatives or causing haplo-insufficiency (32–34) but how exactly expression of mutant alleles results in changes in TNFRSF13B signaling and function is incompletely known (10). In contrast, the number of missense mutations in the *HLA-A* gene is as expected by a sequence-context-based mutational model as (z score of −0.1 according to the Genome Aggregation Database, v2.1.1; http://exac.broadinstitute.org) and the *HLA-A* gene is less tolerant than *TNFRSF13B* to loss of function mutations. Thus, *TNFRSF13B* missense alleles appear to have been selectively retained and recent analysis by the McDonald-Kreitman neutrality index suggests the locus is under strong positive selection (35) in contrast with prior analysis of smaller cohorts (36). This is in contrast to genes encoding HLA which are under moderate purifying pressure (35). Some *TNFRSF13B* polymorphisms are conserved across mammalian species. As an example, mice have 17 missense alleles, 2 non-sense alleles, and 2 splice variants (37).

Adding to TNFRSF13B diversity the receptor is expressed as two isoforms that differ by the presence (long, L) or absence (short, S) of exon 2 that is alternatively spliced following B cell activation (38). The short version of the receptor lacks the cysteine rich domain 1 thought to mediate ligand binding (38). However, absence of the CDR1 domain in the short form does not appear to preclude assembly of the receptor trimer or signaling. Garcia-Carmona et al. (10) showed that TNFRSF13B-S and TNFRSF13B-L assembled receptor complexes composed of one single S or L isoform, or of mixed complexes composed of both S and L isoforms. TNFRSF13B-S requires a lower ligand concentration to signal than TNFRSF13B-L, in part owing to increased ligand binding affinity (10). In contrast, certain mutated isoforms, C104R, A181E, and S194X, produced receptors that had impaired or no signaling (10).

TNFRSF13B polymorphisms may be maintained by balancing selection. Balancing selection is thought to occur when multiple alleles (variants) are maintained in the population in an equilibrium, at frequencies more evenly distributed than expected under models of neutral evolution, because selection favors the heterozygote. To measure the likelihood of balancing selection at the *TNFRSF13B* locus we used a statistic, the β index, which detects clusters in close proximity to a site targeted by balancing selection [https://academic.oup.com/mbe/article/34/11/2996/3988103; (39)]. *TNFRSF13B* manifests balancing selection in four regions (two of them between exons 2 and 3, and the other two between exons 4 and 5) (39). Research by Jagoda et al. (40) suggests that certain haplotypes of *TNFRSF13B* may be of Neanderthal in origin and kept in European/Eurasian populations as a result from adaptive introgression. Thus, *TNFRSF13B* variants may have originated by positive selection of archaic ancestry variants, maintained thereafter by balancing selection.

What the selection pressures are that maintain *TNFRSF13B* diversity is not known but our research and the research of others connects these properties to the control of T-independent natural IgA antibody production.

TNFRSF13B AND IgA

TNFRSF13B promotes IgA synthesis. In support, TNFRSF13B-deficient animals and animals with dominant-negative *TNFRSF13B* alleles are IgA deficient (12, 17, 20) and; selective IgA deficiency in humans is often associated with mutant *TNFRSF13B* alleles (41). We hypothesize that *TNFRSF13B* polymorphisms may in part be driven by the receptor impact on secretory IgA (sIgA).

First discovered in 1953, IgA is by some measures the most abundant Ig in the body (42, 43). IgA exists as a monomer in circulation and as a dimer in lumina of the respiratory, intestinal, and genito-urinary system. Secretory IgA makes 2/3 of all IgA produced in the body (44). An IgA monomer is, like other Ig isotypes, a tetramer composed of two identical heavy chains and two identical light chains united by covalent and non-covalent bonds. Each monomer contains two Fab domains and one Fc region that includes C alpha 2 and C alpha 3 exons (44). Humans, express IgA1 and IgA2, the later encoded by several distinct alleles. IgA1 and IgA2 differ on the hinge region that links constant domain alpha 1 to the constant domain alpha 2

of the heavy chain, longer in IgA1 and shorter on IgA2. The significance of the longer hinge region for the function of IgA1 is not completely known but modeling suggests that the longer hinge region may afford greater flexibility to the variable region relative to the Fc region at the cost of increased sensitivity to proteolysis (44).

The secreted form of IgA is a complex comprised of an IgA dimer linked by the joining (J) chain and a secretory component, a fragment of the polymeric Ig receptor (PIgR). Secretion and dimerization of IgA is made possible by two important adaptations. In one, IgA has an 18 aa tail piece at the C-terminus, highly homologous to another found at the C-terminus of IgM. The tail piece allows IgA (and IgM) polymerization because it binds to the J chain through 2 cysteine disulfide bonds (45, 46). The J chain is a 137 amino-acid polypeptide and it is bound to IgA before secretion. The second adaptation is the development of a highly specific IgA transport system across the epithelium and into secretions. The transport of IgA depends on the polymeric Ig receptor (pIgR) that binds only polymeric Ig (IgA or IgM) and is expressed on the basolateral surface of epithelial cells that line mucosal surfaces of the gut, lung biliary tract, and lacrimal glands. The pIgR has a 620 aa extra-cellular domain, a 23 aa transmembrane domain and a 103 aa cytoplasmic domain (47). Transcytosis of IgA from the submucosal region to the lumen requires binding of dimeric IgA C alpha 3 to the pIgR at the basolateral side of epithelial cells followed by internalization and transport to the apical surface of the cell. IgA is released from its attachment by cleavage of the pIgR ectodomain (secretory component) at the apical surface. IgA, covalently bound to the secretory component is released on to the mucosa, forming sIgA. Bacterial and viral products induce heightened pIgR synthesis by epithelial cells and hence increase transcytosis of IgA (47). The secretory component confers resistance to proteolysis and anti-microbial functions. In addition to binding to IgA, the secretory component exists as a free protein and as such it binds directly to a variety of microbes inhibiting adhesion to epithelial cells, binds to mucus mediating immune exclusion of antigens and pathogens, retains IL-8 and in this way, inhibits neutrophil chemotaxis (48).

IgA complexed with J chain and the secretory component is heavily glycosylated and glycosylation is necessary for many of non-specific, variable region-independent IgA functions (49). IgA complexes are modified by N-linked glycosylation at Asn (N) residues and by O-linked sugars. Glycosylation of the secretory component protects IgA from degradation (50) and mediates some IgA immune functions. Glycans enable secretory component binding to adhesins and lectins, to a wide range of bacteria and to toxins [(48), and references therein]. Glycosylation of the secretory component determines the distribution of IgA to specific areas of the epithelium (51) and the secretory component is needed with IgA to neutralize rotavirus (52). Furthermore, secretory component free or complexed to IgA, binds to the lectin binding domain of Mac-1 (CR3, CD11b/CD18) enhancing IgA receptor Fc alpha signaling and augmenting phagocytosis and inflammation [reviewed in (53)].

J chain glycosylation is necessary for dimerization of IgA (54) and for maintaining the correct conformation to facilitate interaction with pIgR (55, 56). Serum IgA and secretory IgA are differentially glycosylated presumably reflecting differences in the glycosylation machinery in plasma cells of the spleen and bone marrow vis a vis plasma cells in the mucosae (48).

The heavy-chain N-linked glycans help maintain the correct IgA conformation and assist with dimer formation and secretion (57, 58). Although the glycan composition of IgA HCs activate the lectin complement activation pathway by binding mannose binding lectin (MBL), the glycans are shielded by the secretory component at physiologic pH and only become accessible for binding at low pH and perhaps upon engagement with bacterial adhesins which pull the secretory component away from the IgA (48). Once uncovered, IgA HC glycans may also directly bind mannose receptors on phagocytic cells promoting internalization and antigen presentation (59). Secretory IgA HCs are also extensively modified by O-linked glycans which form highly complex structures that in addition to stabilizing the hinge region also interact with bacterial adhesins (60). Glycosylation-dependent IgA functions are likely due to low affinity interactions and therefore critically dependent on IgA concentration. Although there is no evidence in support of TNFRSF13B direct influence on IgA glycosylation, by controlling IgA secretion TNFRSF13B is likely to impact more on the non-specific IgA functions than on the functions of IgA that depend on high affinity interactions between mutated V regions and their targets.

DISCUSSION

One might expect that a cytokine receptor such TNFRSF13B would exert straight forward functions that promote host defense and/or immune regulation and accordingly polymorphism should be rare and harmful. Yet, TNFRSF13B is as diverse as MHC. Why?

Although the story is yet incomplete, the complexity of TNFRSF13B gene and protein functions has been coming more fully into focus. Although TNFRSF13B does not bind antigen it controls response of B cells to T-independent and to T-dependent antigens differently. TNFRSF13B promotes T-independent antibody production in part by facilitating differentiation of plasma cells. In contrast, TNFRSF13B-deficiency does not inhibit and in some cases enhances IgG or IgA responses to T-dependent antigens.

Most sIgA results from T-independent B cell responses. IgA exerts protective functions by helping to eliminate pathogens directly and/or through maintaining microbial homeostasis and these functions are in a great extent independent of IgA specificity (61–63). The equilibrium established between IgA, microbes and pathogens results from anti-microbial functions of IgA and microbial adaptations to IgA (49, 64). In one example of a remarkable microbial adaptation, Nakajima et al. (49) showed that highly glycosylated IgA of an irrelevant specificity (specific to ovalbumin) bound to a human symbiont *Bacteroides thetaiotaomicron* (*B. theta*) changing gene expression. IgA induced transcription of *B. theta* polysaccharide utilization loci (65) changing polysaccharide utilization and in this way

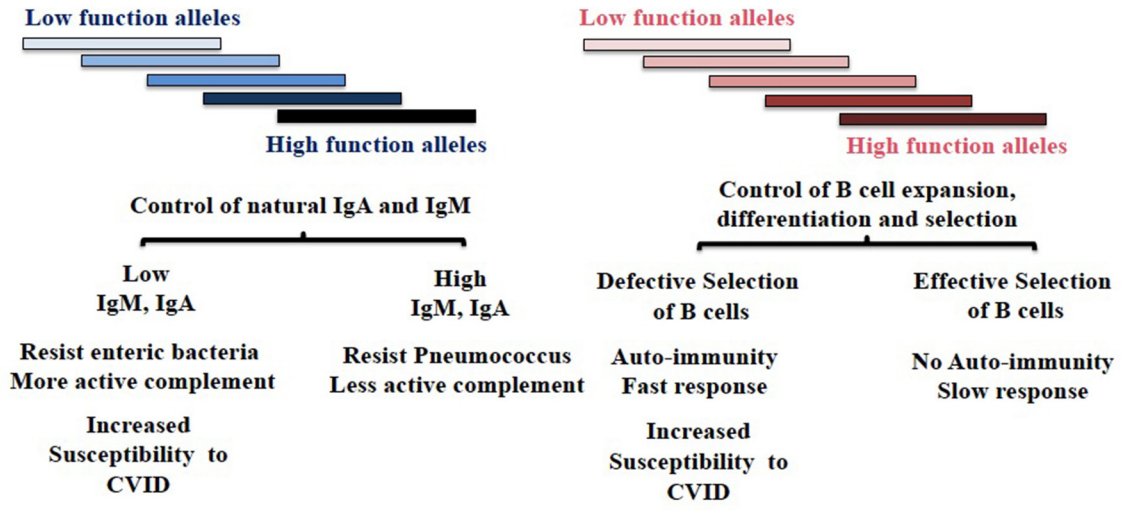

FIGURE 1 | Diversity of *TNFRSF13B* in host response to environmental challenges. *TNFRSF13B* encodes a receptor (TACI) through to support the maturation of B cells, yet the gene is among the most polymorphic in the genome and polymorphism is maintained across mammalian species. The authors speculate that polymorphism of *TNFRSF13B* reflects balancing selection—balancing benefits and disadvantages of signals the receptor delivers but also manifold intermediate functions the protein and its neighbors in cell membrane confer. The figure depicts two hypothetically distinct regions of the protein that confer phenotypically distinct properties. The balancing of complex sets of phenotypic properties that presumably impact all mammals could underlie extraordinary diversity of the gene across species. CVID, Common Variable Immunodeficiency.

conferring competitive advantage (49). As another example, *Bacteroides fragilis* capsule induces polysaccharide specific IgA which in turn, increases its adherence to intestinal epithelial cells by binding to mucus as well as to the capsule polysaccharides (62). In these examples, commensal bacteria co-opt IgA to colonize the gut.

We hypothesize *TNFRSF13B* polymorphisms are maintained at least in part as the result of microbial adaptation to IgA (**Figure 1**). We offer the following reasoning in support of the hypothesis.

In a recent report Grasset et al. (18) showed that TNFRSF13B is necessary for T-independent IgA responses to commensal bacteria. These responses include secretion of polyreactive IgA and represent the majority of secreted IgA. In contrast, T cell-dependent IgA responses occur independently of TNFRSF13B. Thus, varying TNFRSF13B function changes the ratio between highly-specific and polyreactive or non-specific IgA responses which, in turn, may counteract pathogen adaptation to IgA and facilitate specific elimination. In support, Grasset et al. (18) showed that *tnfrsf13b-KO* mice generated mutated IgA that specifically targeted a restricted subset of microbes. In an earlier publication, Tsuji et al. (17) showed that *tnfrsf13b-KO* produced highly mutated antibodies that rapidly cleared *C. rodentium*. Whether changes of the properties and amount of sIgA and/or varying the proportion of mixed polyreactive and specific mono-reactive Ig in individuals or mice expressing *TNFRSF13B* polymorphisms is protective awaits investigation.

For all the benefits of IgA, selective IgA deficiency is often asymptomatic (41). *TNFRSF13B* polymorphisms are associated with IgA deficiency (41), the most common immune-deficiency (with frequencies varying between 1:143 in the Arabian peninsula to 1 in 500 Caucasian individuals) (66, 67). The limited morbidity of IgA deficiency, suggests as one possibility that benefits conferred by TNFRSF13B variants may outweigh the detrimental impact of decreased IgA in the gut. Because *tnfrsf13b* mutants maintain the ability to make "adaptive" IgA it is possible that protective functions of IgA are maintained or even enhanced. In accord, IgA-deficient patients were found to have enhanced adaptive antibody responses to pneumococcal vaccination (68). Perhaps it is this type of response that explains the mild phenotype of many individuals with IgA deficiency and the maintenance of the extreme polymorphism at the *TNFRSF13B* locus.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Animal studies and human subject research that inspired some of the hypotheses in the manuscript were reviewed and approved by the Institutional Animal Care & Use Committee (IACUC) or by the Institutional Review Board (IRB) at the University of Michigan.

AUTHOR CONTRIBUTIONS

MC and JP wrote the manuscript. MC analyzed data. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Cyster JG, Allen CDC. B cell responses: cell interaction dynamics and decisions. *Cell.* (2019) 177:524–40. doi: 10.1016/j.cell.2019.03.016
2. Elgueta R, de Vries VC, Noelle RJ. The immortality of humoral immunity. *Immunol Rev.* (2010) 236:139–50. doi: 10.1111/j.1600-065X.2010.00924.x
3. Bischof D, Elsawa SF, Mantchev G, Yoon J, Michels GE, Nilson A, et al. Selective activation of TACI by syndecan-2. *Blood.* (2006) 107:3235–42. doi: 10.1182/blood-2005-01-0256
4. Xia XZ, Treanor J, Senaldi G, Khare SD, Boone T, Kelley M, et al. TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. *J Exp Med.* (2000) 192:137–43. doi: 10.1084/jem.192.1.137
5. Bonilla FA, Geha RS. Common variable immunodeficiency. *Pediatr Res.* (2009) 65(5 Pt 2):13R−9. doi: 10.1203/PDR.0b013e31819dbf88
6. von Bulow GU, Bram RJ. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. *Science.* (1997) 278:138–41.
7. Ozcan E, Rauter I, Garibyan L, Dillon SR, Geha RS. Toll-like receptor 9, transmembrane activator and calcium-modulating cyclophilin ligand interactor, and CD40 synergize in causing B-cell activation. *J Allergy Clin Immunol.* (2011) 128:601–9.e1-4. doi: 10.1016/j.jaci.2011.04.052
8. Allman WR, Dey R, Liu L, Siddiqui S, Coleman AS, Bhattacharya P, et al. TACI deficiency leads to alternatively activated macrophage phenotype and susceptibility to Leishmania infection. *Proc Natl Acad Sci USA.* (2015) 112:E4094–103. doi: 10.1073/pnas.1421580112
9. He B, Santamaria R, Xu W, Cols M, Chen K, Puga I, et al. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat Immunol.* (2010) 11:836–45. doi: 10.1038/ni.1914
10. Garcia-Carmona Y, Ting AT, Radigan L, Athuluri Divakar SK, Chavez J, Meffre E, et al. TACI isoforms regulate ligand binding and receptor function. *Front Immunol.* (2018) 9:2125. doi: 10.3389/fimmu.2018.02125
11. Sintes J, Gentile M, Zhang S, Garcia-Carmona Y, Magri G, Cassis L, et al. mTOR intersects antibody-inducing signals from TACI in marginal zone B cells. *Nat Commun.* (2017) 8:1462. doi: 10.1038/s41467-017-01602-4
12. Tsuji S, Cortesao C, Bram RJ, Platt JL, Cascalho M. TACI deficiency impairs sustained Blimp-1 expression in B cells decreasing long-lived plasma cells in the bone marrow. *Blood.* (2011) 118:5832–9. doi: 10.1182/blood-2011-05-353961
13. Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, Schlesier M, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat Genet.* (2005) 37:820–8. doi: 10.1038/ng1600
14. Castigli E, Wilson SA, Garibyan L, Rachid R, Bonilla F, Schneider L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet.* (2005) 37:829–34. doi: 10.1038/ng1601
15. Kralickova P, Milota T, Litzman J, Malkusova I, Jilek D, Petanova J, et al. CVID-associated tumors: czech nationwide study focused on epidemiology, immunology, and genetic background in a cohort of patients with CVID. *Front Immunol.* (2018) 9:3135. doi: 10.3389/fimmu.2018.03135
16. Romberg N, Chamberlain N, Saadoun D, Gentile M, Kinnunen T, Ng YS, et al. CVID-associated TACI mutations affect autoreactive B cell selection and activation. *J Clin Invest.* (2013) 123:4283–93. doi: 10.1172/JCI69854
17. Tsuji S, Stein L, Kamada N, Nunez G, Bram R, Vallance BA, et al. TACI deficiency enhances antibody avidity and clearance of an intestinal pathogen. *J Clin Invest.* (2014) 124:4857–66. doi: 10.1172/JCI74428
18. Grasset EK, Chorny A, Casas-Recasens S, Gutzeit C, Bongers G, Thomsen I, et al. Gut T cell-independent IgA responses to commensal bacteria require engagement of the TACI receptor on B cells. *Sci Immunol.* (2020) 5:eaat7117. doi: 10.1126/sciimmunol.aat7117
19. Kanswal S, Katsenelson N, Selvapandiyan A, Bram RJ, Akkoyunlu M. Deficient TACI expression on B lymphocytes of newborn mice leads to defective Ig secretion in response to BAFF or APRIL. *J Immunol.* (2008) 181:976–90. doi: 10.4049/jimmunol.181.2.976
20. Jabara HH, Lee JJ, Janssen E, Ullas S, Liadaki K, Garibyan L, et al. Heterozygosity for transmembrane activator and calcium modulator ligand interactor A144E causes haploinsufficiency and pneumococcal susceptibility in mice. *J Allergy Clin Immunol.* (2017) 139:1293–301.e4. doi: 10.1016/j.jaci.2016.07.028
21. Roque R, Ponce R, Burleson F, Cabrit M, Broly H, Rogge M. Influenza virus host response of C57Bl/6 mice treated with TACI-Ig. *Immunopharmacol Immunotoxicol.* (2006) 28:13–32. doi: 10.1080/08923970600623541
22. Wolf AI, Mozdzanowska K, Quinn WJ, 3rd, Metzgar M, Williams KL, Caton AJ, et al. Protective antiviral antibody responses in a mouse model of influenza virus infection require TACI. *J Clin Invest.* (2011) 121:3954–64. doi: 10.1172/JCI57362DS1
23. Salzer U, Bacchelli C, Buckridge S, Pan-Hammarstrom Q, Jennings S, Lougaris V, et al. Relevance of biallelic versus monoallelic TNFRSF13B mutations in distinguishing disease-causing from risk-increasing TNFRSF13B variants in antibody deficiency syndromes. *Blood.* (2009) 113:1967–76. doi: 10.1182/blood-2008-02-141937
24. von Bulow GU, van Deursen JM, Bram RJ. Regulation of the T-independent humoral response by TACI. *Immunity.* (2001) 14:573–82. doi: 10.1016/S1074-7613(01)00130-3
25. Mantchev GT, Cortesao C, Rebrovich M, Cascalho M, Bram RJ. TACI is required for efficient plasma cell differentiation in response to T-independent type 2 antigens. *J Immunol.* (2007) 179:2282–8. doi: 10.4049/jimmunol.179.4.2282
26. Ding BB, Bi E, Chen H, Yu JJ, Ye BH. IL-21 and CD40L synergistically promote plasma cell differentiation through upregulation of Blimp-1 in human B cells. *J Immunol.* (2013) 190:1827–36. doi: 10.4049/jimmunol.1201678
27. Sakurai D, Kanno Y, Hase H, Kojima H, Okumura K, Kobata T. TACI attenuates antibody production costimulated by BAFF-R and CD40. *Eur J Immunol.* (2007) 37:110–8. doi: 10.1002/eji.200636623
28. Romberg N, Virdee M, Chamberlain N, Oe T, Schickel JN, Perkins T, et al. TNF receptor superfamily member 13b (TNFRSF13B) hemizygosity reveals transmembrane activator and CAML interactor haploinsufficiency at later stages of B-cell development. *J Allergy Clin Immunol.* (2015) 136:1315–25. doi: 10.1016/j.jaci.2015.05.012
29. Radwan J, Babik W, Kaufman J, Lenz TL, Winternitz J. Advances in the evolutionary understanding of MHC polymorphism. *Trends Genet.* (2020) 36:298–311. doi: 10.1016/j.tig.2020.01.008
30. Castigli E, Wilson SA, Scott S, Dedeoglu F, Xu S, Lam KP, et al. TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med.* (2005) 201:35–9. doi: 10.1084/jem.20032000
31. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature.* (2016) 536:285–91. doi: 10.1038/nature19057
32. Garibyan L, Lobito AA, Siegel RM, Call ME, Wucherpfennig KW, Geha RS. Dominant-negative effect of the heterozygous C104R TACI mutation in common variable immunodeficiency (CVID). *J Clin Invest.* (2007) 117:1550–7. doi: 10.1172/JCI31023

33. Lee JJ, Jabara HH, Garibyan L, Rauter I, Sannikova T, Dillon SR, et al. The C104R mutant impairs the function of transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) through haploinsufficiency. *J Allergy Clin Immunol.* (2010) 126:1234–41.e2. doi: 10.1016/j.jaci.2010.08.017
34. Lee JJ, Rauter I, Garibyan L, Ozcan E, Sannikova T, Dillon SR, et al. The murine equivalent of the A181E TACI mutation associated with common variable immunodeficiency severely impairs B-cell function. *Blood.* (2009) 114:2254–62. doi: 10.1182/blood-2008-11-189720
35. Quintana-Murci L. Human immunology through the lens of evolutionary genetics. *Cell.* (2019) 177:184–99. doi: 10.1016/j.cell.2019.02.033
36. Sazzini M, Zuntini R, Farjadian S, Quinti I, Ricci G, Romeo G, et al. An evolutionary approach to the medical implications of the tumor necrosis factor receptor superfamily member 13B (TNFRSF13B) gene. *Genes Immunity.* (2009) 10:566–78. doi: 10.1038/gene.2009.43
37. Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, et al. Ensembl (2018). *Nucleic Acids Res.* (2018) 46:D754–61. doi: 10.1093/nar/gkx1098
38. Garcia-Carmona Y, Cols M, Ting AT, Radigan L, Yuk FJ, Zhang L, et al. Differential induction of plasma cells by isoforms of human TACI. *Blood.* (2015) 125:1749–58. doi: 10.1182/blood-2014-05-575845
39. Siewert KM, Voight BF. Detecting long-term balancing selection using allele frequency correlation. *Mol Biol Evol.* (2017) 34:2996–3005. doi: 10.1093/molbev/msx209
40. Jagoda E, Lawson DJ, Wall JD, Lambert D, Muller C, Westaway M, et al. Disentangling Immediate adaptive introgression from selection on standing introgressed variation in humans. *Mol Biol Evol.* (2018) 35:623–30. doi: 10.1093/molbev/msx314
41. Vo Ngoc DT, Krist L, van Overveld FJ, Rijkers GT. The long and winding road to IgA deficiency: causes and consequences. *Expert Rev Clin Immunol.* (2017) 13:371–82. doi: 10.1080/1744666X.2017.1248410
42. Cunningham-Rundles C. Physiology of IgA and IgA deficiency. *J Clin Immunol.* (2001) 21:303–9. doi: 10.1023/A:1012241117984
43. Grabar P, Williams CA. [Method permitting the combined study of the electrophoretic and the immunochemical properties of protein mixtures; application to blood serum]. *Biochim Biophys Acta.* (1953) 10:193–4. doi: 10.1016/0006-3002(53)90233-9
44. de Sousa-Pereira P, Woof JM. IgA: structure, function, and developability. *Antibodies.* (2019) 8:57. doi: 10.3390/antib8040057
45. Frutiger S, Hughes GJ, Paquet N, Luthy R, Jaton JC. Disulfide bond assignment in human J chain and its covalent pairing with immunoglobulin M. *Biochemistry.* (1992) 31:12643–7. doi: 10.1021/bi00165a014
46. Hamburger AE, Bjorkman PJ, Herr AB. Structural insights into antibody-mediated mucosal immunity. *Curr Top Microbiol Immunol.* (2006) 308:173–204. doi: 10.1007/3-540-30657-9_8
47. Kaetzel CS. The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunol Rev.* (2005) 206:83–99. doi: 10.1111/j.0105-2896.2005.00278.x
48. Royle L, Roos A, Harvey DJ, Wormald MR, van Gijlswijk-Janssen D, Redwan el RM, et al. Secretory IgA N- and O-glycans provide a link between the innate and adaptive immune systems. *J Biol Chem.* (2003) 278:20140–53. doi: 10.1074/jbc.M301436200
49. Nakajima A, Vogelzang A, Maruya M, Miyajima M, Murata M, Son A, et al. IgA regulates the composition and metabolic function of gut microbiota by promoting symbiosis between bacteria. *J Exp Med.* (2018) 215:2019–34. doi: 10.1084/jem.20180427
50. Crottet P, Corthesy B. Secretory component delays the conversion of secretory IgA into antigen-binding competent F(ab')2: a possible implication for mucosal defense. *J Immunol.* (1998) 161:5445–53.
51. Phalipon A, Cardona A, Kraehenbuhl JP, Edelman L, Sansonetti PJ, Corthesy B. Secretory component: a new role in secretory IgA-mediated immune exclusion *in vivo*. *Immunity.* (2002) 17:107–15. doi: 10.1016/S1074-7613(02)00341-2
52. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. *Science.* (1996) 272:104–7. doi: 10.1126/science.272.5258.104
53. van Egmond M, Damen CA, van Spriel AB, Vidarsson G, van Garderen E, van de Winkel JG. IgA and the IgA Fc receptor. *Trends Immunol.* (2001) 22:205–11. doi: 10.1016/S1471-4906(01)01873-7
54. Krugmann S, Pleass RJ, Atkin JD, Woof JM. Structural requirements for assembly of dimeric IgA probed by site-directed mutagenesis of J chain and a cysteine residue of the alpha-chain CH2 domain. *J Immunol.* (1997) 159:244–9.
55. Vaerman JP, Langendries A, Giffroy D, Brandtzaeg P, Kobayashi K. Lack of SC/pIgR-mediated epithelial transport of a human polymeric IgA devoid of J chain: *in vitro* and *in vivo* studies. *Immunology.* (1998) 95:90–6.
56. Vaerman JP, Langendries AE, Giffroy DA, Kaetzel CS, Fiani CM, Moro I, et al. Antibody against the human J chain inhibits polymeric Ig receptor-mediated biliary and epithelial transport of human polymeric IgA. *Eur J Immunol.* (1998) 28:171–82.
57. Taylor AK, Wall R. Selective removal of alpha heavy-chain glycosylation sites causes immunoglobulin A degradation and reduced secretion. *Mol Cell Biol.* (1988) 8:4197–203.
58. Atkin JD, Pleass RJ, Owens RJ, Woof JM. Mutagenesis of the human IgA1 heavy chain tailpiece that prevents dimer assembly. *J Immunol.* (1996) 157:156–9.
59. Heystek HC, Moulon C, Woltman AM, Garonne P, van Kooten C. Human immature dendritic cells efficiently bind and take up secretory IgA without the induction of maturation. *J Immunol.* (2002) 168:102–7. doi: 10.4049/jimmunol.168.1.102
60. Bos NA, Bun JC, Popma SH, Cebra ER, Deenen GJ, van der Cammen MJ, et al. Monoclonal immunoglobulin A derived from peritoneal B cells is encoded by both germ line and somatically mutated VH genes and is reactive with commensal bacteria. *Infect Immun.* (1996) 64:616–23. doi: 10.1128/IAI.64.2.616-623.1996
61. Catanzaro JR, Strauss JD, Bielecka A, Porto AF, Lobo FM, Urban A, et al. IgA-deficient humans exhibit gut microbiota dysbiosis despite secretion of compensatory IgM. *Sci Rep.* (2019) 9:13574. doi: 10.1038/s41598-019-49923-2
62. Donaldson GP, Ladinsky MS, Yu KB, Sanders JG, Yoo BB, Chou WC, et al. Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science.* (2018) 360:795–800. doi: 10.1126/science.aaq0926
63. Macpherson AJ, Yilmaz B, Limenitakis JP, Ganal-Vonarburg SC. IgA function in relation to the intestinal microbiota. *Annu Rev Immunol.* (2018) 36:359–81. doi: 10.1146/annurev-immunol-042617-053238
64. Mathias A, Corthesy B. Recognition of gram-positive intestinal bacteria by hybridoma- and colostrum-derived secretory immunoglobulin A is mediated by carbohydrates. *J Biol Chem.* (2011) 286:17239–47. doi: 10.1074/jbc.M110.209015
65. Martens EC, Chiang HC, Gordon JI. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe.* (2008) 4:447–57. doi: 10.1016/j.chom.2008.09.007
66. Yel L. Selective IgA deficiency. *J Clin Immunol.* (2010) 30:10–6. doi: 10.1007/s10875-009-9357-x
67. Wang N, Hammarstrom L. IgA deficiency: what is new? *Curr Opin Allergy Clin Immunol.* (2012) 12:602–8. doi: 10.1097/ACI.0b013e3283594219
68. Edwards E, Razvi S, Cunningham-Rundles C. IgA deficiency: clinical correlates and responses to pneumococcal vaccine. *Clin Immunol.* (2004) 111:93–7. doi: 10.1016/j.clim.2003.12.005

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Chemokine Receptor-6 Promotes B-1 Cell Trafficking to Perivascular Adipose Tissue, Local IgM Production and Atheroprotection

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Chemokine receptor-6 (CCR6) mediates immune cell recruitment to inflammatory sites and has cell type-specific effects on diet-induced atherosclerosis in mice. Previously we showed that loss of CCR6 in B cells resulted in loss of B cell-mediated atheroprotection, although the B cell subtype mediating this effect was unknown. Perivascular adipose tissue (PVAT) harbors high numbers of B cells including atheroprotective IgM secreting B-1 cells. Production of IgM antibodies is a major mechanism whereby B-1 cells limit atherosclerosis development. Yet whether CCR6 regulates B-1 cell number and production of IgM in the PVAT is unknown. In this present study, flow cytometry experiments demonstrated that both B-1 and B-2 cells express CCR6, albeit at a higher frequency in B-2 cells in both humans and mice. Nevertheless, B-2 cell numbers in peritoneal cavity (PerC), spleen, bone marrow and PVAT were no different in $ApoE^{-/-}CCR6^{-/-}$ compared to $ApoE^{-/-}CCR6^{+/+}$ mice. In contrast, the numbers of atheroprotective IgM secreting B-1 cells were significantly lower in the PVAT of $ApoE^{-/-}CCR6^{-/-}$ compared to $ApoE^{-/-}CCR6^{+/+}$ mice. Surprisingly, adoptive transfer (AT) of $CD43^{-}$ splenic B cells into B cell-deficient $\mu MT^{-/-}ApoE^{-/-}$ mice repopulated the PerC with B-1 and B-2 cells and reduced atherosclerosis when transferred into $ApoE^{-/-}CCR6^{+/+}sIgM^{-/-}$ mice only when those cells expressed both CCR6 and sIgM. CCR6 expression on circulating human B cells in subjects with a high level of atherosclerosis in their coronary arteries was lower only in the putative human B-1 cells. These results provide evidence that B-1 cell CCR6 expression enhances B-1 cell number and IgM secretion in PVAT to provide atheroprotection in mice and suggest potential human relevance to our murine findings.

Keywords: B-1 cells, IgM, CCR6, atherosclerosis, inflammation, perivascular adipose tissue

INTRODUCTION

Atherosclerosis is well-recognized as a chronic inflammatory disease of arteries and plaque rupture is the primary underlying cause of cardiovascular events. Atherosclerosis develops when low density lipoproteins (LDL) enter the subendothelial layer of the artery wall and become oxidized. Products of oxidized lipids are highly reactive and modify self-molecules, thereby generating oxidation-specific epitopes (OSE) that are recognized by receptors of the immune system, including scavenger receptors on macrophages leading to foam cell formation. Oxidized LDL and foam cells promote inflammatory cytokine production and induce the expression of cell adhesion molecules on endothelial cells. Surface adhesion molecule expression recruits inflammatory cells such as monocytes, T cells, natural killer cells, natural killer T cells, and dendritic cells into the subendothelial layer, developing lesion formation (1–3).

B cells play a major role in the regulation of atherosclerosis, and their effects are subset and context-dependent. B-1 B cells attenuate and B-2 B cells aggravate atherosclerosis (4–9). Treatments that deplete predominantly B-2 cells such as anti-CD20 monoclonal antibody and B-cell activating factor receptor (BAFFR) deficiency attenuated atherosclerosis development in apolipoprotein-E deficient ($ApoE^{-/-}$) and low-density lipoprotein receptor deficient ($LdlR^{-/-}$) mice maintained on Western diet (WD) (5, 6, 8, 9). In contrast, IgM secreting B-1 cells have been shown to be atheroprotective (4, 7). B-1 cells, which are the major source for circulatory IgM (10, 11) and attenuate atherosclerosis (4, 7, 8), can be further divided into two sister populations: B-1a and B-1b. IgM can block the uptake of oxidized LDL by macrophages (12) and, immunization to boost IgM to OSE resulted in atheroprotection (13).

Perivascular adipose tissue (PVAT) directly contacts the artery adventitia and has a role in the regulation of atherosclerosis. Data demonstrates that PVAT adjacent to atherosclerotic human blood vessels is more inflamed than PVAT adjacent to non-diseased vessel segments (14). Adipocytes in PVAT secrete both pro inflammatory and anti-inflammatory cytokines (15). We have recently shown that the PVAT harbors both B-1 and B-2 B cell subtypes in young $ApoE^{-/-}$ mice and these local B-1 cells can be induced to proliferate by cytokines (16) and produce atheroprotective IgM antibodies locally (17). However, the mechanism involved in B-1 cell trafficking to PVAT is not known.

Chemokines and chemokine receptors regulate immune cell trafficking and may be key targets or molecules to control homing of immune cells to sites of human disease (18). C-C chemokine receptor 6 (CCR6) is a G protein-coupled receptor expressed on different immune cell types such as macrophages, immature dendritic cells, T and B-lymphocytes (19). CCR6 and its ligand CCL20 have emerged as important regulators of atherosclerosis (18, 20–22). Global deletion of *Ccr6* in $ApoE^{-/-}$ and $LdlR^{-/-}$ mice resulted in decreased Ly6C^{hi} monocyte exit from the bone marrow, less recruitment of pro-atherogenic macrophages to lesions and attenuated atherosclerosis (20, 22). In human atherosclerosis, the expression of CCR6 and CCL20 in atherosclerotic lesions in the coronary and carotid arteries has been reported. In addition, CCL20 expression is positively correlated with DC numbers in the shoulder regions of the lesion (23), and this DC derived CCL20 may attract CCR6 expressing immune cells into the lesion. Also, circulating levels of CCL20 is significantly increased in hypercholesterolemic patients and LDL stimulated vascular smooth muscle cells express CCL20 and promote human lymphocyte migration (18), implicating a role for the CCR6-CCL20 axis in atherosclerosis development. Previous data from our lab demonstrated that CCR6 regulated aortic homing of CD43^{-} splenocytes (B cells) and diet induced atheroprotection in B cell deficient ($\mu MT^{-/-}$) mice (21). In general, CD43^{-} splenocytes are thought to be B-2 cells which are considered atherogenic. Thus, it is not clear how the aortic homing of these CD43^{-} splenocytes provides atheroprotection. B cell subsets may have distinct functions in the local PVAT compared to peripheral sites, and atheroprotection may depend on the ability of B cells to home to athero-prone aortic sites (21). Taken together, the results suggest that CCR6 may be a pro- or anti-atherogenic chemokine receptor depending on the cell type in which it is expressed and underscore a need to better understand the role of CCR6 in B cells.

In this current study, we provide the first evidence of a B-1 cell specific function for CCR6 in regulating B-1 numbers and IgM production in PVAT. We demonstrate that atheroprotective effects of CD43^{-} splenocytes in $ApoE^{-/-}sIgM^{-/-}$ mice are CCR6-dependent and require the cells to be capable of secreting IgM implicating PVAT trafficking of B-1 cells as a potentially important atheroprotective mechanism. Further, we demonstrate that in humans, expression of CCR6 on a putative B-1 cell population (24) was significantly reduced in patients with a high degree of coronary artery disease (CAD) underscoring the potential clinical relevance of our findings and suggesting that B-1 cell-specific augmentation of CCR6 expression may be a potential therapeutic approach.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. Apolipoprotein E deficient ($ApoE^{-/-}$) mice, B cell deficient ($\mu MT^{-/-}$) mice, and chemokine receptor six deficient ($CCR6^{-/-}$) were purchased from Jackson Laboratory and maintained in our animal facility (University of Virginia). $\mu MT^{-/-}$ and $CCR6^{-/-}$ mice were bred to the $ApoE^{-/-}$ line to develop $ApoE^{-/-}\mu MT^{-/-}$ and $ApoE^{-/-}CCR6^{-/-}$ knockouts. Generated $ApoE^{-/-}CCR6^{+/-}$ mice and setup heterozygous breeders ($ApoE^{-/-}CCR6^{+/-}$ *x* $ApoE^{-/-}CCR6^{+/-}$) to generate $ApoE^{-/-}CCR6^{-/-}$ and their littermate controls $ApoE^{-/-}CCR6^{+/+}$. All purchased mice were on C57BL/6J background and those bred were backcrossed to C57BL/6J mice for 10 generations. $sIgM^{-/-}$ mice were kindly provided by Dr. Peter Lobo (University of Virginia) and crossed to $ApoE^{-/-}$ mice to generate $ApoE^{-/-}sIgM^{-/-}$ mice. All mice were given water *ad libitum* and standard chow diet (Tekland, 7012). Mice were euthanized with CO_2 inhalation. Young (8–10 weeks) male mice were used for all experiments except for atherosclerosis studies. For atherosclerosis studies,

ApoE$^{-/-}$ mice were maintained on WD (42% fat, Tekland, 88137) for 12 weeks.

Human Samples

Patients ($n = 118$) were recruited for study through the Cardiac Catheterization laboratory at the University of Virginia as previously described (25). All participants provided written informed consent prior to enrollment, and the study was approved by the Human IRB Committee at UVA. A total of 118 patients presenting for a medically indicated diagnostic cardiac catheterization were enrolled if they met inclusion criteria. Patients were excluded if they had: any acute illness, type 1 diabetes, current acute coronary syndrome, autoimmune disease or on immunosuppressive therapy, prior organ transplantation, anemia, pregnancy, or HIV infection. No patient was on anticoagulation or had deep vein thrombosis or pulmonary embolism. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood for flow cytometry experiments. Isolation of PBMC's from human peripheral blood was performed by RBC lysis in whole blood, and the purified PBMCs were used for flow staining. List of antibodies for cell surface markers (name of the clone) for human flow cytometry: CD3 (SK7 or UCHT1), CD20 (2H7), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (61D3), CCR6 (11A9), CD27 (M-T271) and CD43 (1G10) were purchased from eBioscience and BD Bioscience. Live/Dead discrimination was determined by LIVE/DEAD fixable yellow staining (Invitrogen). Cells were run on a CyAN ADP (Beckman Coulter). Data were analyzed with FlowJo 9 software.

Coronary Artery Disease Severity Measured by Gensini Score

Gensini Score (GS) is a widely used angiographic scoring system to measure the severity of coronary artery disease (CAD) (26). We used quartiles to categorize patients with CAD based on GS. Participants with scores in the first three quartiles (GS: 0–33.25) were categorized in the low GS group ($n = 80$) and participants with scores in fourth quartiles (GS: 33.25–128) were categorized as in the high GS group ($n = 38$). Quartile values were calculated using a larger cohort in which the current cohort is nested.

Flow Cytometry

Peritoneal cavity lavage (PerC), spleen and bone marrow (BM) cells were harvested and single cell suspensions were prepared as previously described (27). In brief, cell suspension from spleen was prepared using a 70 μm cell strainer and mashing spleen with a syringe plunger, and dissolved in FACS buffer. To isolate BM cells, femur and tibia were collected and flushed with FACS buffer. Spleen and BM samples were re-suspended in erythrocyte lysis buffer and washed. Stromal vascular fraction was prepared from PVAT as previously described (27). In brief, to harvest PVAT, first, para aortic lymph nodes were carefully removed and then PVAT was carefully harvested and weighed. PVAT was collected into 5 mL FACS tubes separately, 2 mL of freshly prepared enzyme cocktail mixture [Collagenase I (450 U/ml) (Sigma), Collagenase XI (125 U/ml) (Sigma), Hyaluronidase I (60 U/ml) (Sigma), DNase (60 U/ml) (Sigma) in PBS with 20 mM HEPES] was added per sample. PVAT was chopped into small pieces and then incubated in a shaking incubator at 37°C for 45 min to obtain single cell suspensions.

For flow staining, cells were blocked for Fc receptors by Fc block (CD16/32) for 10 min on ice, and were stained for cell surface markers using fluorescently conjugated antibodies for 30 min on ice. After washing and centrifugation, cells were washed and stained with a fixable live/dead stain diluted in PBS for 15 min on ice and then fixed in 2% PFA in PBS for 10 min at room temperature prior to re-suspending in FACS buffer. Flow cytometry antibodies: CD19 (1D3), B220/CD45R (RA3-6B2), CD5 (53-7.3), CD43 (S7), CCR6 (29-2L17) and IgM (II/41, R6-60.2) were purchased from eBioscience, BD Bioscience, and Biolegend. Live/Dead discrimination was determined by LIVE/DEAD fixable yellow staining (Invitrogen). Cells were run on CyAN ADP (Beckman Coulter) and Attune NxT flow cytometer (Invitrogen). Data were analyzed with FlowJo 10 software.

Adoptive Transfer Experiments

PerC cells were isolated from *ApoE*$^{-/-}$ mice and stained for FACSorting (InFlux sorter). Cells were stained with fluorescence labeled antibodies against CD19 (1D3), B220 (RA3-6B2), CD23 (B3B4), IgD (11-26c) and CD5 (53-7.3), and live cells were gated for DAPI^{-} population. Two hundred thousand of FACSorted B-1 cells were adoptively transferred into 8–10 week old *ApoE*$^{-/-}$s*IgM*$^{-/-}$ mice via intraperitoneal injection. For CD43^{-} splenocyte isolation, spleens were harvested from 10–12 week old donor mice and B cells were isolated using MACS anti-CD43 microbeads (Miltenyi Biotec) as per the manufacturer's protocol. The purity of representative samples was analyzed by flow cytometry and found to be >97%. Either 30×10^6 or 60×10^6 B cells were resuspended in 200 μl of PBS and adoptively transferred *via* tail vein injection to 8–10 week old recipients.

Enzyme-Linked ImmunoSpot Assay

Single cell suspensions of PVAT, spleen and BM were prepared as described above in the flow cytometry section. ELISPOT was performed as previously described (4, 17, 27). Sterile MultiScreen IP-Plates (Millipore, MSIPS4510) were used for the assay according to manufacturer's protocol. Wells were coated with unlabeled goat anti-mouse IgM antibody (10 μg/ml; Southern Biotech) and incubated overnight at 4°C. The next day, antibody solution was decanted, membrane was washed with PBS and then blocked with RPMI 1640+10% FCS for 2 h at 37°C. A suspension of 1×10^6 cells/ml was prepared in ice cold culture media for spleen and BM from which 100,000 cells were plated for each of the sample as starting concentration and then were serially diluted in subsequent wells. For PVAT samples, resuspended in 250 μl culture media and were used as starting concentration from which serial dilutions in subsequent wells were prepared. The plate was incubated overnight at 37 °C in a cell culture incubator (5% CO_2). Cells were decanted, washed (PBS+0.01% tween-20) and incubated with biotin-labeled goat anti-mouse IgM antibody (1:500 dilution) (Southern Biotech) for 2 h in a cell culture incubator. After washing, cells were incubated for 30 min at room temperature in streptavidin alkaline phosphatase (Abcam). Again, following washing BCIP/NBT (Gene Tex Inc.)

was added and incubated until spots became visible. Each spot on the membrane indicated an antibody secreting cell. Spots were counted manually.

Enzyme-Linked Immunosorbent Assay

Plasma or serum samples were collected from mice and circulatory total IgM and IgG levels were quantified by ELISA as published before (28). Total IgE ELISA was performed according to manufacturer's guidelines (BioLegend). For MDA epitope specific IgM and IgG ELISA, we used Peptide mimotope for malondialdehyde (MDA) epitope (P2 peptide-biotin) (Peptide 2.0) as a capture antigen (29). Briefly, 96 well microtiter plates (Corning) were incubated at 4°C overnight with capture un labeled IgM or IgG diluted in coating buffer (0.1 M disodium phosphate pH 9.0). Plates were blocked (PBS containing 0.5% BSA, 0.1% TWEEN-20, and 0.01% NaN_3), incubated with samples, and then treated with IgM detection antibody conjugated to alkaline phosphatase for 2 h at room temperature. Detection antibodies and dilutions used: murine IgM-AP and murine IgG-AP (Southern Biotech). Plates were then developed with pNPP solution (Southern Biotech) for 30–60 min and read at 405 nm using a SpectraMax 190 (Molecular Devices). IgM concentration was determined through a standard curve of purified immunoglobulin (Southern Biotech) using a range of 0.098–200 ng/ml. All dilutions were determined through careful titration, and only values within the range of standard curves with readings at least 3-fold higher than negative controls were used.

Enface Staining

Aortas were harvested carefully as previously described (17). Aortas were opened longitudinally, fixed in 4% formaldehyde, pinned, and stained with Sudan IV (Sigma). Aortas were imaged with a Nikon D70 DSLR camera and enface lesion area was quantified using ImagePro Plus 7.0 software.

Statistics

Student's *t*-test was used for analyzing data with normal distribution and equal variance. For data sets with non-normal distribution, Wilcoxon rank-sum test was used. Similarly, a non-parametric test (Spearman's correlation) was used to correlate data that was not normally distributed. One-way Anova with multiple comparison was used when compared multiple groups. Results are displayed containing all replicated experiments, and values shown are mean ± SEM. Data were analyzed using Prism 8 (GraphPad Software, Inc) and SAS (SAS version 9.4).

RESULTS

A Higher Frequency of CCR6$^+$ B-1 Cells in Tissue Compartments That Support Antibody Production Compared to Their Homeostatic Niche

To compare the expression levels of CCR6 in B-1 and B-2 cells, we performed flow cytometry on isolated cells from various compartments and measured the frequency of CCR6$^+$ B-1 and B-2 cells from atherosclerosis prone chow fed $ApoE^{-/-}$ mice. **Figure 1A** depicts the gating strategy for identifying B-1 and B-2 cells and a representative histogram showing CCR6 expression relative to an FMO control. Quantification of the frequency of CCR6$^+$ B-1 and B-2 cells in different tissue compartments revealed that both B-1 and B-2 cells express CCR6. Interestingly, the frequency of CCR6$^+$ B-1 cells was significantly higher in antibody secreting tissue compartments such as spleen, BM and PVAT compared to the primary B-1 cell niche, PerC (**Figure 1B**). However, despite a higher frequency of CCR6$^+$ cells, there was no such difference observed in B-2 cells (**Figure 1C**). This data suggests that CCR6 may be important for B-1 cell recruitment to antibody secreting regions.

CCR6 Deficiency Reduces B-1 Cell Numbers in PVAT

Previous data from our lab clearly showed that CCR6 regulates B cell migration to aorta and provides atheroprotection (21) and that PVAT harbors higher numbers of atheroprotective IgM-producing B-1 cells than the aorta itself (17). To determine the effect of CCR6 deficiency on B cell subset distribution in PVAT and other tissue compartments in atherosclerosis-prone mice at homeostasis, flow cytometry and ELISPOT experiments were performed in young $ApoE^{-/-}CCR6^{+/+}$ and $ApoE^{-/-}CCR6^{-/-}$ littermate control mice (10 weeks old) fed normal Chow diet. There was no difference in total body weight (**Figure 2A**) and PVAT weight (**Figure 2B**). PVAT collected from the aortic arch to the iliac bifurcation was carefully dissected and flow cytometry was performed to analyze B-1 and B-2 cells (**Figure 2C**). Flow cytometry data revealed B-1 cell numbers but not B-2 cell numbers were significantly reduced in PVAT of $ApoE^{-/-}CCR6^{-/-}$ mice compared to $ApoE^{-/-}CCR6^{+/+}$ mice (**Figure 2D**). ELISPOT data demonstrate that total IgM secreting B cells were reduced in PVAT in $ApoE^{-/-}CCR6^{-/-}$ mice compared to $ApoE^{-/-}CCR6^{+/+}$ mice (**Figure 2E**). Intriguingly, there was no difference of B-1 and B-2 cell numbers in PerC, spleen and BM compartments between $ApoE^{-/-}CCR6^{+/+}$ and $ApoE^{-/-}CCR6^{-/-}$ mice (**Figures 2F–H**). Correspondingly, there was no difference in total IgM secreting B cells in spleen and BM $ApoE^{-/-}CCR6^{+/+}$ and $ApoE^{-/-}CCR6^{-/-}$ mice (**Figure 2I**), and there was no difference in plasma total IgM levels between $ApoE^{-/-}CCR6^{+/+}$ and $ApoE^{-/-}CCR6^{-/-}$ mice as well (**Figure 2J**).

CD43$^-$ Splenocytes (B cells) Can Repopulate/Establish B-1 Cell Compartment

To understand whether B-1 cells can migrate, survive and produce IgM in PVAT, FACS-sorted B-1 cells (2×10^5) were adoptively transferred into secretory IgM ($sIgM^{-/-}$) deficient $ApoE^{-/-}$ mice via i.p injection. After 7 weeks of the adoptive transfer, plasma IgM levels were measured by ELISA at different time points. The plasma IgM levels were boosted after 1st week of the B-1 cell transfer compared to PBS injected $ApoE^{-/-}sIgM^{-/-}$ mice. However, secreted IgM levels in the plasma eventually dropped down to baseline within 4 weeks of transfer (**Supplementary Figure 1**). Flowcytometry analysis confirmed non-survival of adoptively transferred B-1 cells in $ApoE^{-/-}sIgM^{-/-}$ mice after 2 weeks (data not shown).

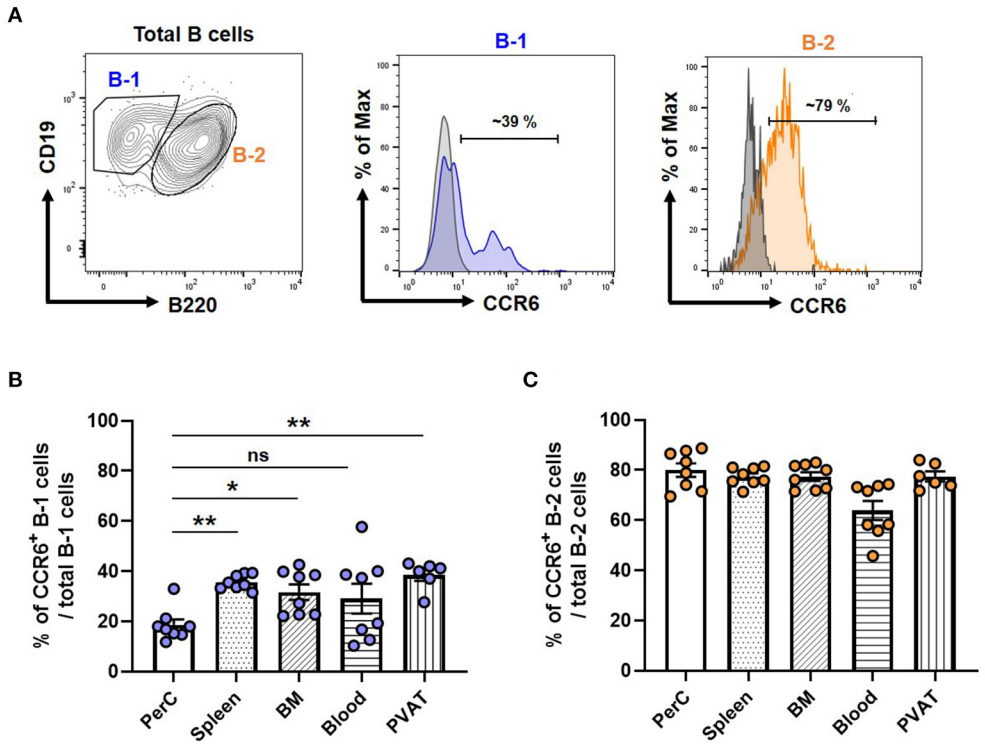

FIGURE 1 | Frequency of CCR6$^+$ B-1 cells was higher in antibody secreting tissue compartments in mice. **(A)** Gating strategy for B-1 and B-2 cells in PVAT and representative histogram of CCR6 expression. Gray line represents FMO for CCR6 and filled blue line represents B-1 or filled orange line represents B-2 cells in flow cytometry histogram plots **(B)** percentage of CCR6$^+$ B-1 cells from total B-1 cells and **(C)** percentage of CCR6$^+$ B-2 cells from total B-2 cells in different tissue compartments. Results are represented in mean ± SEM, Performed One-way ANOVA with multiple comparisons. *$p < 0.05$ and **$p < 0.01$. $n = 6$–8 mice/group and each dot represents individual mouse.

Since survival of B-1 cells alone is not possible for long-term experiment we sought out an alternative approach.

We previously showed that adoptive transfer of CD43$^-$ splenocytes (B cells) into B cell deficient mice provided atheroprotection compared to vehicle (PBS) injected mice (21). Yet, CD43$^-$ splenocytes have been thought to represent B-2 cells which are considered atherogenic (5, 6, 8, 9) and B-1 cells are CD43$^+$ (10, 17, 30). However, this paradigm did not explain how CD43$^-$ B cell transfer provided atheroprotection in B cell deficient *ApoE*$^{-/-}$ mice (*ApoE*$^{-/-}\mu MT^{-/-}$) after 16 weeks of WD (21), raising an important question whether there could be B-1 cells in the CD43$^-$ compartment. We analyzed CD43 expression in B cell compartments by doing flow cytometry in *ApoE*$^{-/-}$ mice. Flow data clearly demonstrate that the majority (>95%) of B-2 cells and ∼10-15 % of B-1a were CD43$^-$ in both PerC and spleen. Interestingly, ∼60% of B-1b cells were CD43$^-$ in both PerC and spleen (**Figure 3A**). The data suggested that the CD43$^-$ splenocyte population contains remnant B-1 cells, in particular B-1b cells. Next, to address whether CD43$^-$ splenocytes can repopulate B-1 cells in recipient mice, MACS purified CD43$^-$ splenocytes (B cells; 60 × 10^6) were adoptively transferred via tail vein injection into *ApoE*$^{-/-}\mu MT^{-/-}$ mice and maintained on WD for 16 weeks (**Figure 3B**). Interestingly, flow cytometry data clearly demonstrated that adoptively transferred CD43$^-$ splenocytes could repopulate both B-1 and B-2 cells in PerC and spleen respectively in *ApoE*$^{-/-}\mu MT^{-/-}$ mice. In addition, these B-1 cells contain both B-1a and B-1b subsets (**Figure 3C**). Also, flow cytometry data confirmed that the purity of CD43$^-$ splenocytes after MACS separation was >97%. MACS separation significantly reduced CD43$^+$ B-1 compartment (∼ 90 %) (**Supplementary Figure 2**). It is also possible that the remnant CD43$^+$ cells could also repopulate. We next wanted to determine if these CD43$^-$ splenocytes in the absence of CCR6 can provide IgM mediated atheroprotection.

Atheroprotection by IgM Secreting B Cells Is CCR6 Dependent

To determine the effect of CCR6 on IgM secreting B cell mediated atheroprotection, 30 × 10^6 CD43$^-$ splenocytes from *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{+/+}$ or *ApoE*$^{-/-}$*CCR6*$^{-/-}$*sIgM*$^{+/+}$ or *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{-/-}$ or PBS (control) mice were adoptively transferred via tail vein injection into *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{-/-}$ mice following which the recipients were fed WD for 12 weeks (**Figure 4A**). Cholesterol levels were no different between the groups after 12 weeks of WD (**Supplementary Figure 3**). We started by determining the

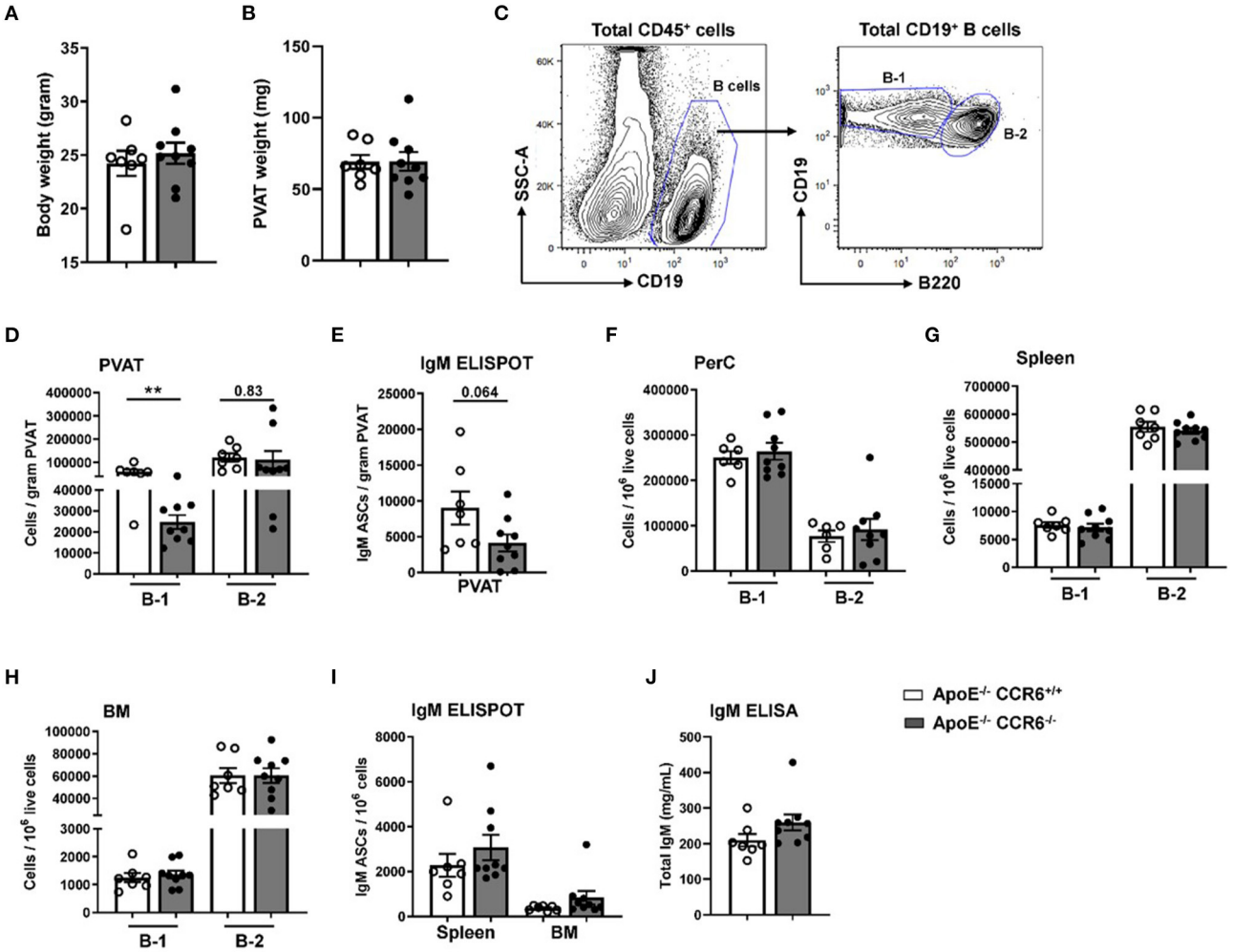

FIGURE 2 | IgM secreting B-1 cells were reduced in PVAT of *ApoE*$^{-/-}$ *CCR6*$^{-/-}$ mice. Comparison of *ApoE*$^{-/-}$*CCR6*$^{+/+}$ and *ApoE*$^{-/-}$*CCR6*$^{-/-}$ littermate control mice showed no difference in **(A)** body weight or **(B)** PVAT weights between groups. **(C)** Gating strategy for total B cells, B-1 and B-2 cells in PVAT. Quantification of **(D)** total B-1 and B-2 cells and **(E)** IgM secreting cells in PVAT. **(F–H)** Quantification of total B-1 and B-2 cells in PerC **(F)**, spleen **(G)** and BM **(H)** and IgM secreting cells in **(I)** spleen and BM. **(J)** quantification of total IgM levels in plasma. Results are represented in mean ± SEM, unpaired student *t*-test was performed. $^{**}p < 0.01$. $n = 7$–9 mice/group and each dot represents individual mouse.

effect of CCR6 on IgM production. We measured circulatory IgM levels by ELISA. Interestingly, circulatory IgM levels were significantly higher in mice that received B cells from *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{+/+}$ mice but not in mice that received CD43^{-} splenocytes from *ApoE*$^{-/-}$*CCR6*$^{-/-}$*sIgM*$^{+/+}$ mice compared to PBS (control) mice. While these differences are detectable in *sIgM*$^{-/-}$ mice, these levels are far lower than in WT mice, likely obscuring this small but significant difference. As expected, there was no difference of circulatory IgM levels in mice that received CD43^{-} splenocytes from *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{-/-}$ mice compared to PBS (control) mice (**Figure 4B**). A trend of lower levels of circulatory IgM and MDA (oxidation specific epitope) mimotope specific IgM were observed in recipients given *ApoE*$^{-/-}$*CCR6*$^{-/-}$*sIgM*$^{+/+}$ CD43^{-} splenocytes, compared to those given *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{+/+}$ CD43^{-} splenocytes (**Figures 4B,C**). No difference in circulatory total IgG, IgE and MDA mimotope specific IgG were observed between PBS (control) and B cell recipient groups (**Supplementary Figure 4**). Next, we wanted to measure atherosclerosis levels. The percentage of enface lesion area was quantified in aortas by Sudan IV staining (**Figure 4D**). Intriguingly, atherosclerosis levels were significantly reduced in mice that received CD43^{-} splenocytes from *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{+/+}$ mice compared to those that received PBS (control). There was no difference in atherosclerosis levels in mice that received CD43^{-} splenocytes from *ApoE*$^{-/-}$*CCR6*$^{-/-}$*sIgM*$^{+/+}$ or *ApoE*$^{-/-}$*CCR6*$^{+/+}$*sIgM*$^{-/-}$ compared to PBS (control). Moreover, the CD43^{-} splenocytes from *ApoE*$^{-/-}$*CCR6*$^{+/+}$ mice attenuated atherosclerosis only when they were capable of secreting IgM (**Figure 4E**).

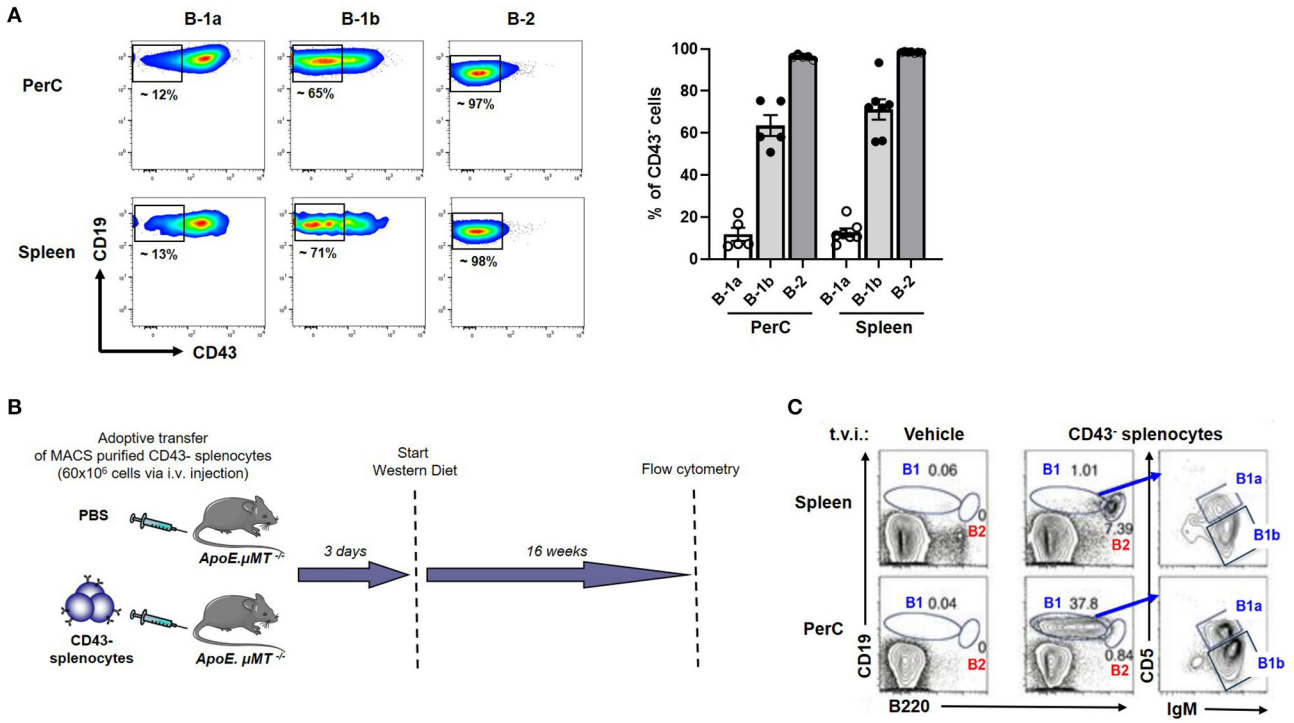

FIGURE 3 | CD43^{-} splenocytes (B cells) contains a CD43^{-} B-1 compartment, predominantly B-1b. **(A)** flow cytometric analysis for CD43^{+} B-1a, B-1b and B-2 cells in PerC and spleen of young *ApoE*$^{-/-}$ mice (n = 5–7). Each dot represents individual mouse. **(B)** Schematic representation of experimental design. CD43^{-} splenocytes from *ApoE*$^{-/-}$ mice or vehicle control were tail vein injected (t.v.i.) into *μMT*$^{-/-}$*ApoE*$^{-/-}$ mice. **(C)** CD43^{-} splenocytes reconstituted PerC B-1 cells and splenic B-2 cells in *μMT*$^{-/-}$*ApoE*$^{-/-}$ mice.

Expression of CCR6 on Human Putative B-1 Cells (CD20^{+} CD27^{+} CD43^{+}) Was Significantly Lower in Patients With High CAD Severity Scores

Next, to examine CCR6 expression on circulatory B cell subsets including putative B-1 cells in humans, PBMCs were collected from patients presenting for a medically indicated coronary angiography CAD assessment. Flow cytometry was performed on PBMCs with antibodies that allowed quantification of CCR6 on B cells, T cells and monocytes. Consistent with previous reports (31–34), all B cells express CCR6 and ∼27% of CD4^{+} T cells and ∼ 10% of CD8^{+} T cells express CCR6. However, circulating human monocytes do not express CCR6 (**Supplementary Figure 5**), despite CCR6 on monocytes being implicated in atherosclerosis in murine models (20, 22). Next to understand the CCR6 expression levels on B-1 cells, total CD20^{+} B cells were gated for naïve, memory and B-1 cells by using CD43 and CD27 (**Figure 5A**) (24, 35). The frequency of CCR6^{+} B-1 cells (**Figure 5B**) and GM mean of CCR6 expression on CD20^{+}CD27^{+}CD43^{+} B-1 cells (**Figure 5C**) was lower compared to naïve and memory B cells in circulation.

To determine whether expression of CCR6 on circulating B cell subtypes was associated with the severity of CAD, we utilized the well-established gensini scoring system (GS) as outlined in the section Methods. GM of CCR6 on total B cells and B cell subsets was quantified in low GS and high GS patients. There was no difference in age, gender, diabetes and hypertension between GS low and high groups (**Supplementary Table 1**). There was no difference of GM of CCR6 on total B cells, naïve and memory B cells (**Figures 5D–F**). Interestingly, GM of CCR6 on B-1 cells was significantly lower in patients with high GS (**Figure 5G**), suggesting that CCR6 expression on human B-1 cells may protect from development of severe coronary artery atherosclerosis.

DISCUSSION

The abundance of atheroprotective B-1 cells in the PVAT has been previously reported by our group (17). However, the mechanism and the role of chemokine receptors in their homing to the PVAT has not been demonstrated. Chemokine receptors are important for the recruitment or migration of immune cells to lymphoid tissues and inflammatory sites to regulate the immune responses. CCR6 is expressed on different leukocyte populations, such as immature dendritic cells (36), B cells (32, 33), T cells (34), NKT cells (37). Previous studies of CCR6 on B cells mainly focused on mature adaptive B-2 cells. Our study is the first to identify a role for CCR6 in regulating B-1 cell number and IgM production in PVAT and a CCR6-dependent IgM-mediated inhibition of diet-induced atherosclerosis.

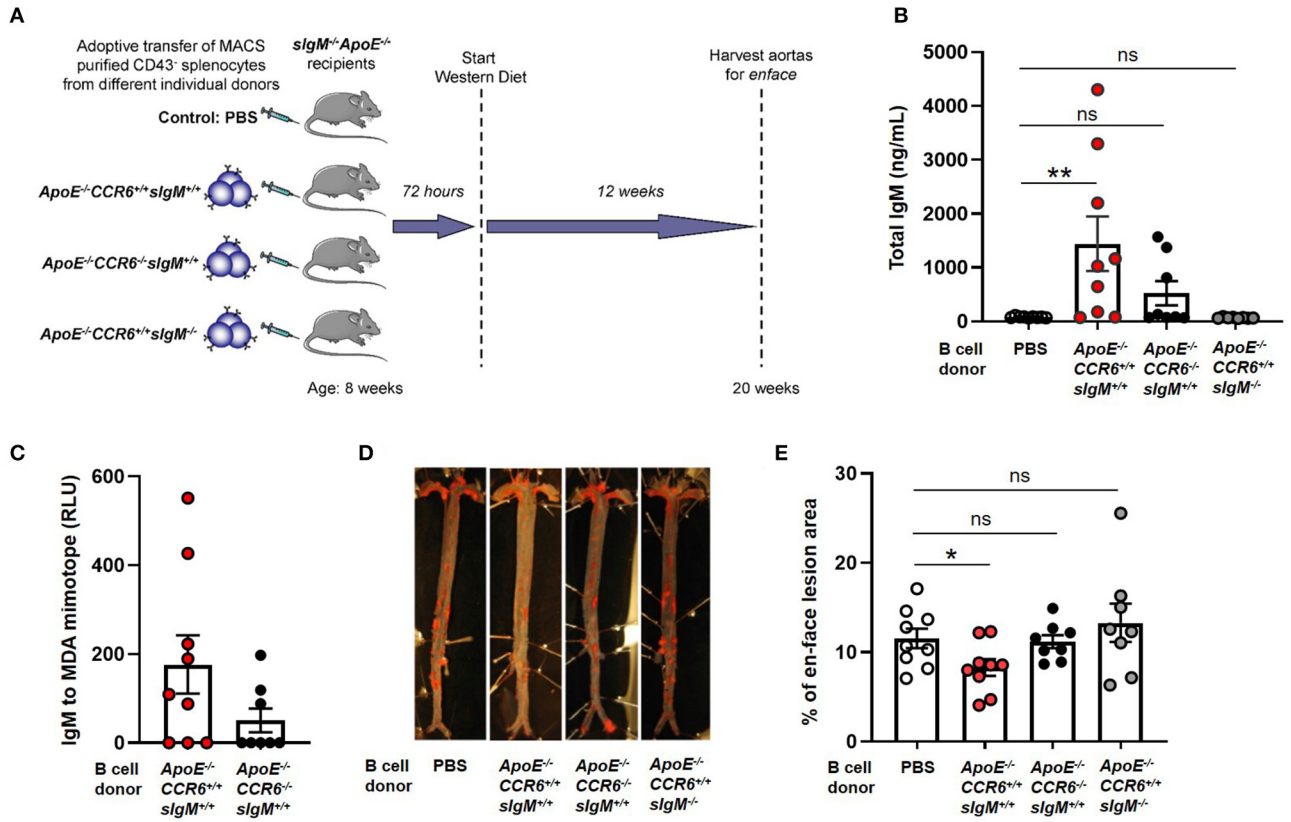

FIGURE 4 | CCR6 expression on IgM secreting B cells is important to provide atheroprotection. **(A)** Schematic representation of experimental design. **(B)** quantification of plasma IgM levels, **(C)** MDA mimotope specific IgM, **(D)** *en-face* staining of aortas and **(E)** quantification of atherosclerosis levels in $sIgM^{-/-}$ $ApoE^{-/-}$ mice after adoptive transfer of $CD43^{-}$ splenocytes from different genotypic donor mice followed by 12 weeks of WD feeding. Results are represented in mean ± SEM, Mann-Whitney unpaired *t*-test was performed. $^{*}p < 0.05$, $^{**}p < 0.01$. $n = 8$–9 mice/group and each dot represents individual mouse.

Two decades ago, an elegant study was published by Krzysiek et al., where they demonstrated the functional role of CCR6 expression during B cell development and antigen mediated B cell differentiation in humans. CCR6 is not expressed during early B cell development in the BM. However, it is expressed later by all mature B cells in the BM, peripheral blood and umbilical cord blood. Eventually, mature B cells lose their CCR6 expression upon activation via B cell receptor signaling and entering into germinal center reactions and terminally differentiating into antibody secreting plasma cells in secondary lymphoid organs. Interestingly, CCR6 is re-expressed once these germinal center B cells differentiate into memory B cells (33) and also these cells show increased chemotactic response to the CCR6 ligand, CCL20 (38). This data suggests that CCR6 expression is restricted to functionally mature B cells capable of responding to antigen challenge. Another study demonstrated that CCR6 expression was important for the migration of memory B cells to the mucosal tissue to produce IgA against intestinal microbial antigens (39). CCR6 is necessary for positioning of memory B cells in spleen to mount recall responses to the same antigen (40) and is thus involved in the regulation of B cell development and their function, particularly, mature activated B-2 cells.

Consistent with previous reports (31, 33), our flow cytometry data in human PBMCs showed that almost all B cells, a small percentage of T cells, and none of the monocytes in circulation express CCR6. In addition, our human data shows that the frequency of $CCR6^{+}$ B-1 cells was significantly lower compared to naïve, and memory B cells in circulation. Though the frequency of $CCR6^{+}$ B-1 cells was lower than B-2 cells in all tissue compartments in the murine system at homeostasis, the frequency of $CCR6^{+}$ B-1 cells in antibody secreting tissue compartments such as the spleen, BM and PVAT was higher compared to their primary homeostatic niche (PerC). Further, in the absence of CCR6 ($ApoE^{-/-}CCR6^{-/-}$) there were significantly fewer IgM secreting B-1 cells in PVAT but not in PerC, spleen and BM. This data suggests that CCR6 expression on B-1 cells may play an important role in B-1 recruitment to PVAT to attenuate disease progression.

Our murine data showed that the number of atheroprotective IgM secreting B-1 cells were significantly reduced in PVAT of $ApoE^{-/-}CCR6^{-/-}$ compared to control group at homeostasis. However, there is no difference in plasma IgM levels between $ApoE^{-/-}CCR6^{+/+}$ and $ApoE^{-/-}CCR6^{-/-}$. This may be due to lack of differences in B-1 cell numbers in the major IgM

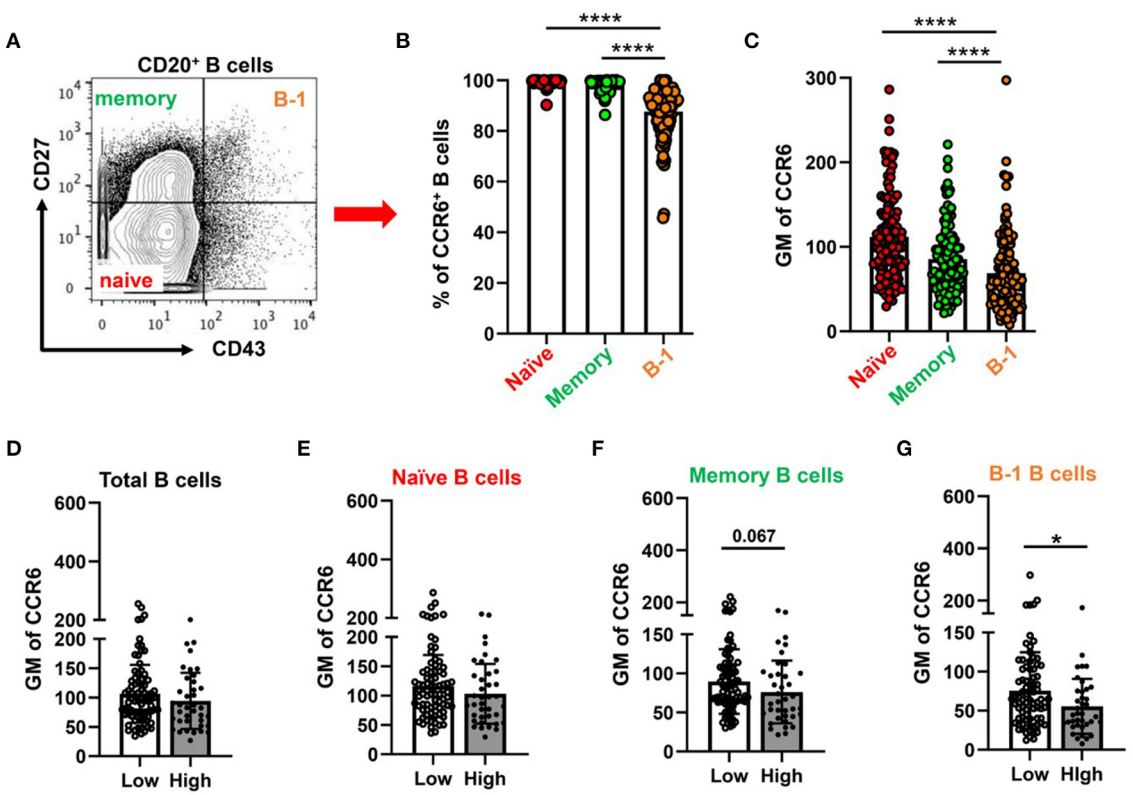

FIGURE 5 | Percentage of CCR6$^+$ and GM of CCR6 on human circulating B cell subtype with lower CCR6 GM on putative human B-1 cells from subjects with high CAD severity score. **(A)** gating strategy for circulating B cell subsets (naïve, memory and B-1 B cells) by using CD43 and CD27. **(B)** percentage of CCR6$^+$ B cell subsets from total B cells and **(C)** GM of CCR6 expression on B cell subsets. Results are shown in mean ± SEM, paired *t*-test was performed. ****$p < 0.0001$. **(D–G)** GM of CCR6 on total B cells **(D)**, naïve **(E)**, memory **(F)** and B-1 B cells **(G)** in subjects with low and high Gensini scores. Results are shown in mean ± SEM, Mann-Whitney *t*-test was performed. *$p < 0.05$.

producing sites such as spleen and BM. Similar to the role of chemokine receptors such as CXCR5, which is important for recruitment/migration of B-1 cells to their primary niches such as PerC and omental fat (41) and CXCR4 in regulation of atheroprotective IgM secreting B-1a cell recruitment to bone marrow (35), our findings provide evidence that CCR6 is important for B-1 cell recruitment to PVAT. The atheroprotective effect is likely due to local (PVAT) IgM secretion, not detected by changes in plasma levels.

Murine studies demonstrate that CCR6 on monocytes is important for their recruitment to aorta to aggravate atherosclerosis. In CCR6 deficient *ApoE*$^{-/-}$ mice atherosclerosis levels were significantly reduced compared to littermate controls (*ApoE*$^{-/-}$*CCR6*$^{+/+}$) after WD feeding which can be attributed to reduction in BM derived Ly6C^{hi} inflammatory monocyte subtype in circulation and thereby reduction in macrophage numbers in atherosclerotic lesions (20). A similar finding was reported in CCR6 deficient *LdlR*$^{-/-}$ mice where atherosclerosis levels were significantly reduced in aorta and aortic sinus *via* reduction in the numbers of Gr-1^{hi} and Gr-1^{low} monocytes in circulation followed by reduction in accumulation of macrophage numbers in the lesions when compared to *LdlR*$^{-/-}$*CCR6*$^{+/+}$ control mice. Contrary to the changes observed in monocyte subset numbers, there was no difference in frequencies of Th1, Th17 and regulatory T cells locally and systemically between *LdlR*$^{-/-}$*CCR6*$^{-/-}$ and *LdlR*$^{-/-}$*CCR6*$^{+/+}$ mice (22). Consistent with our human data on CCR6 expression on human leukocytes, initially, CCR6 was not thought to be expressed on human monocytes (42). However, studies have shown a substantial increase in CCR6 expression on monocytes during inflammatory conditions. Around 3% of circulatory monocytes expressed CCR6 in blood and synovial fluid from rheumatoid arthritis patients and these monocytes responded to CCL20 chemotaxis *in vitro* (31). This suggests that chemokine receptor expression may increase on leukocytes during inflammatory conditions and promote recruitment to sites of inflammation in humans.

While global deletion of CCR6 conferred atheroprotection, previous studies of the role of CCR6 on B cells demonstrate an atheroprotective effect. Adoptive transfer of CD43$^-$ splenocytes from CCR6$^{+/+}$ but not CCR6$^{-/-}$ donors showed increased recruitment of B cells to the aorta (taken together with PVAT) and significantly reduced diet induced atherogenesis in $\mu MT^{-/-}$ recipients (21). Also, upon comparing aorta and PVAT separately, we have shown that PVAT and not aorta has more B cells (17). This suggests that CCR6 is involved in B cell homing to PVAT and in conferring B cell-mediated atheroprotection.

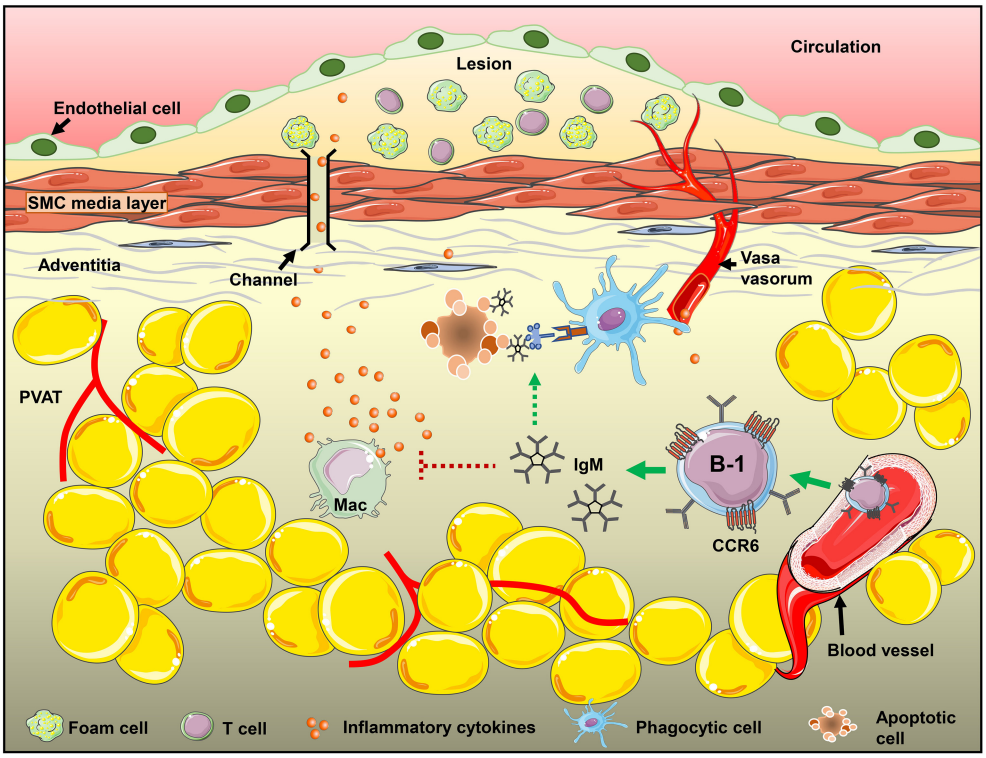

FIGURE 6 | CCR6 expression on B-1 cells regulate B-1 numbers in PVAT and these B-1 cells attenuate diet-induced atherosclerosis in an IgM-dependent manner via inhibiting inflammatory cytokine production from inflammatory macrophages and/or increasing apoptotic cell clearance in the PVAT.

Adoptive transfer of FACS sorted B-1 cells alone do not survive long-term in $ApoE^{-/-}sIgM^{-/-}$ recipients. We therefore took the approach of transferring $CD43^{-}$ splenocytes as per our previous publication (21). Notably, although the percentage of splenic B-1 cells that are $CD43^{-}$ is lower than the percentage of $CD43^{-}$ B-2 cells, 10-15% of B-1a and 60-70% of B-1b cells in both PerC and spleen are $CD43^{-}$ (**Figure 3A**). These B-1 cells together with few remnant $CD43^{+}$ B-1 cells could re-populate during the 16 weeks of time prior to the end of our study due to their self-renewing capacity. Our current study suggests that this re-population of atheroprotective IgM secreting B-1 cells led to atheroprotection.

In this current study we performed the adoptive transfer of $CD43^{-}$ splenocytes into sIgM deficient mice that have endogenous B cells unlike the $uMT^{-/-}$ mice in our previous study (21). sIgM deficiency increases marginal zone B cells and reduces follicular B cells (43, 44). Though marginal zone B cells are primarily involved in providing protection from blood borne pathogens (45). However, these B cells are also involved in atheroprotection by reducing follicular T helper cell development via programmed death ligand-1 signaling (46). These follicular T helper cells are important for differentiation of follicular B cells into germinal center B cells followed by antigen specific antibody producing B cells. IgG is a major antibody type secreted by B cells that undergo germinal center reactions. With regards to follicular B cells, it has been reported that $sIgM^{-/-}$ mice display increased B cell receptor signaling that results in abnormal B cell development followed by reduced follicular B cell numbers (44). This condition leads to increased circulatory pathogenic IgE levels in circulation and aggravated atherosclerosis in atherogenic diet fed $Ldlr^{-/-}sIgM^{-/-}$ mice compared to control group (47). None of the $ApoE^{-/-}sIgM^{-/-}$ recipient mice in our study regardless of genotype of the donor cells had differences in circulatory total IgG and IgE antibody levels arguing against this mechanism for the atheroprotection seen in our study.

We have previously shown that sIgM deficiency aggravates atherosclerosis in mice (48) and local PVAT mRNA levels of proinflammatory cytokines like INFγ and TNFα in $sIgM^{-/-}$ mice are significantly higher compared to littermate controls (unpublished data). This supports the role of locally produced IgM in regulation of PVAT inflammation. This data is consistent with our previous report that show that CCR6 deficiency significantly reduced B cell recruitment to aorta and adoptive transfer of B cells from $CCR6^{-/-}$ mice no longer provided atheroprotection (21). CCR6 is thus important for B-1 cell recruitment and local IgM production in the PVAT. B-1 cell derived IgM in fact could influence the disease levels by altering the local environment in the PVAT of mice either by reducing inflammatory cytokine production (TNFα and MCP-1) from M1 type macrophages in adipose tissue (28), or by mediating opsonization of apoptotic cells and accelerating their clearance by phagocytic cells (49, 50). CCR6, thus, may regulate B-1 cell recruitment to PVAT and control local inflammatory responses

by secreting IgM and thereby its use in development of cell-based therapeutics could help in modulation of the disease (**Figure 6**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Virginia Human Investigation Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University of Virginia Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

PS designed and performed the experiments, acquired and analyzed the data, prepared figures, and wrote the manuscript. AU, HMP, MAM, and CM performed the experiments. FD analyzed human data. SVB edited scientific content in the manuscript. AMT acquired coronary angiography data. CAM designed the experiments and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.636013/full#supplementary-material

Supplementary Figure 1 | B-1 cells do not survive longer periods in sIgM deficient mice: **(A)** schematic representation of experimental design. FACS sorted B-1 cells *ApoE*$^{-/-}$ mice were adoptively transferred into *sIgM*$^{-/-}$*ApoE*$^{-/-}$ mice. After 1 weeks of the transfer, mice were maintained on WD for 6 weeks. **(B)** After blood was collected at different time points and circulatory IgM levels were measured by ELISA (n = 4/group).

Supplementary Figure 2 | FACS confirmation of MACS purified CD43^{-} B cells: CD43^{-} splenocyte isolation was performed by MACS separation. CD43^{+} B cells were shown before MACS and after MACS separation.

Supplementary Figure 3 | No difference in total cholesterol levels. Serum cholesterol levels were measured in *sIgM*$^{-/-}$*ApoE*$^{-/-}$ mice that received PBS and CD43^{-} splenocytes from different genotypic donor mice followed by 12 weeks of WD feeding (**Figure 5A**). There was no difference in serum total cholesterol levels between these groups. Results are represented in mean + SEM, Mann-Whitney unpaired *t*-test was performed. n = 8–9 mice/group and each dot represents individual mouse.

Supplementary Figure 4 | No difference in circulatory IgG levels. Serum **(A)** total IgG, **(B)** MDA mimotope specific IgG, and **(C)** total IgE levels were measured in *sIgM*$^{-/-}$*ApoE*$^{-/-}$ mice that received PBS and CD43^{-} splenocytes from different genotypic donor mice followed by 12 weeks of WD feeding (**Figure 5A**). There was no difference between control (PBS) group and CD43^{-} splenocytes transferred from different genotypic mice groups. Results are represented in mean + SEM, Mann-Whitney unpaired *t*-test was performed. n = 8–9 mice/group and each dot represents individual mouse.

Supplementary Figure 5 | Total B cells and few percent of T cells express CCR6 but not monocytes in PBMCs: Flow cytometry analysis to determine CCR6 expression levels on different circulating immune cells in human. CCR6 highly expressed on B cells and moderately expressed on T cell subsets but no expression on monocytes (n = 15).

REFERENCES

1. Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol.* (2011) 12:204–12. doi: 10.1038/ni.2001
2. Wigren M, Nilsson J, Kolbus D. Lymphocytes in atherosclerosis. *Clin Chim Acta. Oct.* (2012) 9:413:1562–8. doi: 10.1016/j.cca.2012.04.031
3. Binder CJ, Papac-Milicevic N, Witztum JL. Innate sensing of oxidation-specific epitopes in health and disease. *Nat Rev Immunol.* (2016) 16:485–97. doi: 10.1038/nri.2016.63
4. Rosenfeld SM, Perry HM, Gonen A, Prohaska TA, Srikakulapu P, Grewal S, et al. B-1b cells secrete atheroprotective IgM and attenuate atherosclerosis. *Circ Res.* (2015) 117:e28–39. doi: 10.1161/CIRCRESAHA.117.306044
5. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, et al. B cell depletion reduces the development of atherosclerosis in mice. *J Exp Med.* (2010) 207:1579–87. doi: 10.1084/jem.20100155
6. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, et al. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol.* (2010) 185:4410–9. doi: 10.4049/jimmunol.1000033
7. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, et al. B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res.* (2011) 109:830–40. doi: 10.1161/CIRCRESAHA.111.248542
8. Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, et al. Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS ONE.* (2012) 7:e29371. doi: 10.1371/journal.pone.0029371
9. Sage AP, Tsiantoulas D, Baker L, Harrison J, Masters L, Murphy D, et al. BAFF receptor deficiency reduces the development of atherosclerosis in mice-brief report. *Arterioscler Thromb Vasc Biol.* (2012) 32:1573–6. doi: 10.1161/ATVBAHA.111.244731
10. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol.* (2012) 42:120–9. doi: 10.1002/eji.201141890
11. Holodick NE, Vizconde T, Rothstein TL. Splenic B-1a cells expressing CD138 spontaneously secrete large amounts of immunoglobulin in naïve mice. *Front Immunol.* (2014) 5:129. doi: 10.3389/fimmu.2014.00129
12. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, et al. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest.* (1999) 103:117–28. doi: 10.1172/JCI4533

13. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between streptococcus pneumoniae and oxidized ldl. *Nat Med.* (2003) 9:736–43. doi: 10.1038/nm876
14. Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, et al. Production of Chemokines by Perivascular Adipose Tissue: A Role in the Pathogenesis of Atherosclerosis? *Arterioscler Thromb Vasc Biol.* (2005) 25:2594–9. doi: 10.1161/01.ATV.0000188508.40052.35
15. Rajsheker S, Manka D, Blomkalns AL, Chatterjee TK, Stoll LL, Weintraub NL. Crosstalk between perivascular adipose tissue and blood vessels. *Curr Opin Pharmacol.* (2010) 10:191–6. doi: 10.1016/j.coph.2009.11.005
16. Perry HM, Oldham SN, Fahl SP, Que X, Gonen A, Harmon DB, et al. Helix-loop-helix factor inhibitor of differentiation 3 regulates interleukin-5 expression and B-1a B cell proliferation. *Arterioscler Thromb Vasc Biol.* (2013) 33:2771–9. doi: 10.1161/ATVBAHA.113.302571
17. Srikakulapu P, Upadhye A, Rosenfeld SM, Marshall MA, McSkimming C, et al. Perivascular adipose tissue harbors atheroprotective IgM-producing B cells. *Front Physiol.* (2017) 8:719. doi: 10.3389/fphys.2017.00719
18. Calvayrac O, Rodríguez-Calvo R, Alonso J, Orbe J, Martín-Ventura JL, Guadall A, et al. CCL20 is increased in hypercholesterolemic subjects and is upregulated by LDL in vascular smooth muscle cells: role of NF-κB. *Arterioscler Thromb Vasc Biol.* (2011) 31:2733–41. doi: 10.1161/ATVBAHA.111.235721
19. Ito T, Carson WF 4th, Cavassani KA, Connett JM, Kunkel SL. CCR6 as a mediator of immunity in the lung and gut. *Exp Cell Res.* (2011) 317:613–9. doi: 10.1016/j.yexcr.2010.12.018
20. Wan W, Lim JK, Lionakis MS, Rivollier A, McDermott DH, Kelsall BL, et al. Genetic deletion of chemokine receptor CCR6 decreases atherogenesis in ApoE-deficient mice. *Circ Res.* (2011) 109:374–81. doi: 10.1161/CIRCRESAHA.111.242578
21. Doran AC, Lipinski MJ, Oldham SN, Garmey JC, Campbell KA, Skaflen MD, et al. B-cell aortic homing and atheroprotection depend on id3. *Circ Res.* (2012) 110:e1–12. doi: 10.1161/CIRCRESAHA.111.256438
22. Manthey HD, Cochain C, Barnsteiner S, Karshovska E, Pelisek J, Koch M, et al. CCR6 seletively promotes monocyte mediated inflammation and atherogenesis in mice. *Thromb Haemost.* (2013) 110:1267–77. doi: 10.1160/TH13-01-0017
23. Yilmaz A, Lipfert B, Cicha I, Schubert K, Klein M, Raithel D, et al. Accumulation of immune cells and high expression of chemokines/chemokine receptors in the upstream shoulder of atherosclerotic carotid plaques. *Exp Mol Pathol.* (2007) 82:245–55. doi: 10.1016/j.yexmp.2006.10.008
24. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+CD70-. *J Exp Med. Jan.* (2011) 208:67–80. doi: 10.1084/jem.20101499
25. Kothari H, Nguyen AT, Yang X, Hisada Y, Tsimikas S, Mackman N, et al. Association of D-dimer with plaque characteristics and plasma biomarkers of oxidation-specific epitopes in stable subjects with coronary artery disease. *J Cardiovasc Transl Res.* (2018) 11:221–9. doi: 10.1007/s12265-018-9790-4
26. Rampidis GP, Benetos G, Benz DC, Giannopoulos AA, Buechel RR. A guide for Gensini Score calculation. *Atherosclerosis.* (2019) 287:181–3. doi: 10.1016/j.atherosclerosis.2019.05.012
27. Srikakulapu P, Hu D, Yin C, Mohanta SK, Bontha SV, Peng L, et al. Artery tertiary lymphoid organs control multilayered territorialized atherosclerosis b-cell responses in aged ApoE-/- mice. *Arterioscler Thromb Vasc Biol.* (2016) 36:1174–85. doi: 10.1161/ATVBAHA.115.306983
28. Harmon DB, Srikakulapu P, Kaplan JL, Oldham SN, McSkimming C, et al. Protective role for B1b B cells and IgM in obesity-associated inflammation, glucose intolerance and insulin resistance. *Arterioscler Thromb Vasc Biol.* (2016) 36:682–91. doi: 10.1161/ATVBAHA.116.307166
29. Amir S, Hartvigsen K, Gonen A, Leibundgut G, Que X, Jensen-Jerolim E, et al. Peptide mimotopes of malondialdehyde epitopes for clinical applications in cardiovascular disease. *J Lipid Res.* (2012) 53:1316–26. doi: 10.1194/jlr.M025445
30. Wells SM, Kantor AB, Stall AM. CD43 (S7) expression identifies peripheral B cell subsets. *J Immunol.* (1994) 153:5503–15.
31. Ruth JH, Shahrara S, Park CC, Morel JC, Kumar P, Qin S, et al. Role of macrophage inflammatory protein-3alpha and its ligand CCR6 in rheumatoid arthritis. *Lab Invest.* (2003) 83:579–88. doi: 10.1097/01.LAB.0000062854.30195.52
32. Bowman EP, Campbell JJ, Soler D, Dong Z, Manlongat N, Picarella D, et al. Developmental switches in chemokine response profiles during B cell differentiation and maturation. *J Exp Med. Apr.* (2000) 191:1303–18. doi: 10.1084/jem.191.8.1303
33. Krzysiek R, Lefevre EA, Bernard J, Foussat A, Galanaud P, Louache F, et al. Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3alpha/CCL20 in human B cells. *Blood.* (2000) 96:2338–45. doi: 10.1182/blood.V96.7.2338
34. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J Immunol.* (1999) 162:186–94.
35. Upadhye A, Srikakulapu P, Gonen A, Hendrikx S, Perry HM, Nguyen A, et al. Diversification and CXCR4-dependent establishment of the bone marrow b-1a cell pool governs atheroprotective igm production linked to human coronary atherosclerosis. *Circ Res.* (2019) 125:e55–e70. doi: 10.1161/CIRCRESAHA.119.315786
36. Iwasaki A, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J Exp Med.* (2000) 191:1381–94. doi: 10.1084/jem.191.8.1381
37. Kim CH, Johnston B, Butcher EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. *Blood.* (2002) 100:11–6. doi: 10.1182/blood-2001-12-0196
38. Wiede F, Fromm PD, Comerford I, Kara E, Bannan J, Schuh W, et al. CCR6 is transiently upregulated on B cells after activation and modulates the germinal center reaction in the mouse. *Immunol Cell Biol.* (2013) 91:335–9. doi: 10.1038/icb.2013.14
39. Bhattacharya D, Cheah MT, Franco CB, Hosen N, Pin CL, Sha WC, et al. Transcriptional profiling of antigen-dependent murine B cell differentiation and memory formation. *J Immunol.* (2007) 179:6808–19. doi: 10.4049/jimmunol.179.10.6808
40. Elgueta R, Marks E, Nowak E, Menezes S, Benson M, Raman VS, et al. CCR6-dependent positioning of memory B cells is essential for their ability to mount a recall response to antigen. *J Immunol.* (2015) 194:505–13. doi: 10.4049/jimmunol.1401553
41. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity.* (2002) 16:67–76. doi: 10.1016/S1074-7613(01)00257-6
42. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev.* (2003) 14:409–26. doi: 10.1016/S1359-6101(03)00049-2
43. Baker N, Ehrenstein MR. Selection of B lymphocyte subsets is regulated by natural IgM. *J Immunol.* (2002) 169:6686–90. doi: 10.4049/jimmunol.169.12.6686
44. Tsiantoulas D, Kiss M, Bartolin-Gritti B, Bergthaler A, Mallat Z, Jumaa H, et al. Secreted IgM deficiency leads to increased BCR signaling that results in abnormal splenic B cell development. *Sci Rep.* (2017) 7:3540. doi: 10.1038/s41598-017-03688-8
45. Zouali M, Richard Y. Marginal zone B cells, a gatekeeper of innate immunity. *Front Immunol.* (2011) 2:63. doi: 10.3389/fimmu.2011.00063
46. Nus M, Sage AP, Lu Y, Masters L, Lam BYH, Newland S, et al. Marginal zone B cells control the response of follicular helper T cells to a high-cholesterol diet. *Nat Med.* (2017) 23:601–10. doi: 10.1038/nm.4315
47. Tsiantoulas D, Bot I, Ozsvar-Kozma M, Gorderle L, Perkmann T, Hartvigsen K, et al. Increased plasma IgE accelerate atherosclerosis in secreted IgM deficiency. *Circ Res.* (2017) 120:78–84. doi: 10.1161/CIRCRESAHA.116.309606

48. Cherepanova OA, Srikakulapu P, Greene ES, Chaklader M, Haskins RM, McCanna ME, et al. Novel autoimmune IgM antibody attenuates atherosclerosis in IgM deficient low-fat diet-fed, but not western diet-Fed Apoe$^{-/-}$ mice. *Arterioscler Thromb Vasc Biol.* (2020) 40:206–19. doi: 10.1161/ATVBAHA.119.312771
49. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol.* (2009) 182:6031–43. doi: 10.4049/jimmunol.0804191
50. Litvack ML, Post M, Palaniyar N. IgM promotes the clearance of small particles and apoptotic microparticles by macrophages. *PLoS ONE.* (2011) 6:e17223. doi: 10.1371/journal.pone.0017223

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Phenotypic and Functional Characterization of Double Negative B Cells in the Blood of Individuals With Obesity

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We have previously shown that obesity is associated with increased secretion of IgG antibodies with anti-self-reactivity. In this paper, we confirm and extend our previous findings. We show that the plasma of individuals with obesity is enriched in autoimmune antibodies whose levels are positively associated with blood frequencies of the subset of Double Negative (DN) B cells, which is the most pro-inflammatory B cell subset. We also show that DN B cells, significantly increased in the blood of obese versus lean individuals, are characterized by higher expression of immune activation markers and of the transcription factor T-bet, both associated with autoimmunity. The removal of DN B cells from the peripheral B cell pool significantly decreases *in vitro* secretion of anti-self IgG antibodies. These results altogether confirm the crucial role of DN B cells in the secretion of anti-self IgG antibodies in individuals with obesity.

Keywords: aging, B cells, obesity, inflammation, antibody responses

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INTRODUCTION

Obesity, defined as body-mass index (BMI) ≥ 30 kg/m^2 by the Centers for Disease Control and Prevention and the World Health Organization, is a condition associated with chronic low-grade systemic inflammation, known as inflammaging (1). Inflammaging has been shown to induce chronic immune activation (IA), which contributes to functional impairment of immune cells and decreased immunity. Obesity and associated inflammation lead to several debilitating chronic diseases such as type-2 diabetes, cancer, atherosclerosis, and inflammatory bowel disease (2–9).

We have previously shown that obesity is associated with decreased antibody responses to the influenza vaccine and decreased B cell function (10), measured by activation-induced cytidine deaminase (AID) after *in vivo* or *in vitro* stimulation with mitogens, antigens and vaccines. AID is the enzyme that regulates Ig class switch recombination (CSR) and somatic hypermutation (SHM) (11), two processes leading to the generation of high affinity protective antibodies (12–14). The reduced B cell resposes in individuals with obesity are likely due to the fact that B cells from obese individuals, as compared to those from lean individuals, are enriched in memory B cells, and in particular in the subset of Double Negative (DN) B cells, which is the most pro-inflammatory B cell subset (10, 15), reported to be increased in the blood of individuals with inflammatory conditions and diseases. These include aging (16–18), autoimmune diseases such as Rheumatoid Arthritis (19),

Systemic Lupus Erythematosus (SLE) (20, 21), Multiple Sclerosis (22), Alzheimer's disease (23), Sjogren's disease (24) and pemphigus (25). DN B cells have also been reported to be increased in the blood of patients affected by chronic infectious diseases such as HIV (26), Hepatitis C (27) and Malaria (28). These results have suggested that these cells likely expand *in vivo* after chronic exposure to autoantigens or pathogen-derived antigens, leading to the production of autoimmune or protective antibodies, respectively. DN B cells are also expanded in the blood of COVID-19 patients and associated with anti-viral antibody responses and poor clinical outcomes, as recently shown (29).

In this paper, we show that the plasma of individuals with obesity is enriched in anti-self IgG antibodies and we tested three different antigenic specificities: double strand (ds)DNA, malondihyldehyde (MDA) and adipocyte-derived antigens. We chose these antigenic specificities because obesity is associated with increased DNA damage (measured by dsDNA) (30), increased oxidative stress and lipid peroxidation (measured by MDA) (31, 32), and increased fat mass (measured by adipocyte-associated antigens released by the adipose tissue) (33). Plasma levels of these anti-self IgG antibodies are positively associated with blood frequencies of DN B cells. We confirmed our previous findings that the frequencies of DN B cells are increased in the blood of obese versus lean individuals. Moreover, we found that DN B cells show higher expression of IA markers and of the transcription factor T-bet associated with autoimmunity. The removal of DN B cells from the total B cell pool significantly reduced *in vitro* secretion of anti-self IgG antibodies. These results reveal a critical role for DN B cells in the secretion of anti-self IgG antibodies in individuals with obesity.

MATERIALS AND METHODS

Subjects

Experiments were performed using blood isolated from lean (n=20, 30–54 years) and obese (n=20, 27–55 years) adult female individuals, with average body Mass Index (BMI, kg/m^2) 21 ± 1 and 42 ± 3, respectively. The individuals participating in the study were screened for diseases known to alter the immune response or for consumption of medications that could alter the immune response. We excluded subjects with autoimmune diseases, congestive heart failure, cardiovascular disease, chronic renal failure, malignancies, renal or hepatic diseases, infectious disease, trauma or surgery, pregnancy, or documented current substance and/or alcohol abuse.

Study participants provided written informed consent. The study was reviewed and approved by our Institutional Review Board (IRB, protocols 20070481 and 20160542), which reviews all human research conducted under the auspices of the University of Miami.

PBMC Collection

PBMC were collected using Vacutainer CPT tubes (BD 362761) and cryopreserved. PBMC ($1x10^6$/ml) were thawed and cultured in complete medium (c-RPMI, RPMI 1640, supplemented with 10% FCS, 10 μg/ml Pen-Strep, 1 mM Sodium Pyruvate, and 2 x 10^{-5} M 2-ME and 2 mM L-glutamine).

Flow Cytometry

After thawing, PBMC (2 x 10^6/ml) were stained for 20 min at room temperature with the following antibodies: anti-CD45 (BioLegend 368540), anti-CD19 (BD 555415), anti-CD27 (BD 555441), and anti-IgD (BD 555778) to measure naive (IgD+CD27-), IgM memory (IgD+CD27+), switched memory (IgD-CD27+), and DN (IgD-CD27-) B cells. To measure membrane expression of markers associated with IA, B cells were also stained with anti-CD95 (BioLegend 305635), anti-CD21 (BioLegend 354911), anti-CD11c (BioLegend 301625), anti-CD86 (BioLegend 374215), anti-HLADR (BioLegend 307617), anti-PD1 (BioLegend 329907) antibodies. Up to 10^4 events in the B cell gate were acquired on an LSR-Fortessa (BD) and analyzed using FlowJo 10.0.6 software. Single color controls were included in every experiment for compensation. Isotype controls were also used in every experiment to set up the gates.

B Cell Isolation and Stimulation

After thawing, B cells were isolated from PBMC using magnetic CD19 Microbeads (Miltenyi), following manufacturer's instructions. Cell preparations were typically >98% pure. B cells were stimulated in c-RPMI with CpG (InvivoGen ODN2006, 10 μg/ml) for 10 days. Supernatants were collected and IgG specificity was measured by ELISA.

To evaluate the effects of DN B cells on IgG autoantibody secretion, CD19+ B cells isolated with magnetic beads were stained with anti-CD27 and anti-IgD antibodies. DN B cells were sorted out in a Sony SH800 cell sorter. Total B cells and total B cells without DN B cells were stimulated for 10 days with CpG, and supernatants analyzed for IgG autoantibody specificity by ELISA.

RNA Extraction and Quantitative PCR

Total RNA was extracted from unstimulated DN B cells, resuspended in TRIzol, according to the manufacturer's protocol, then resuspended into 10 μl of preheated H_2O, and stored at -80°C until use. Reverse Transcriptase (RT) reactions were performed in a Mastercycler Eppendorf Thermocycler to obtain cDNA. Briefly, 2 μl of RNA at the concentration of 0.5 μg/μl were used as template for cDNA synthesis in the RT reaction. Conditions were: 40 min at 42°C and 5 min at 65°C. Five μl of cDNA were used for qPCR. Reactions were conducted in MicroAmp 96-well plates and run in the ABI 7300 machine. Calculations were made with ABI software. Briefly, we determined the cycle number at which transcripts reached a significant threshold (Ct) for each target gene and for GAPDH as control. A value of the target gene, relative to GAPDH, was calculated and expressed as ΔCt. Reagents and primers (Taqman) were from ThermoFisher.

ELISA to Measure Antibodies in Plasma and Culture Supernatants

For dsDNA-specific and Malondihyldehyde (MDA)-specific IgG antibodies we used the Signosis EA-5002 and MyBioSource MBS390120 kits, respectively.

For adipocyte-specific IgG antibodies, we isolated the adipocytes from the subcutaneous adipose tissue of patients undergoing weight reduction surgeries (bilateral breast reduction), as previously described (33). After isolation, the adipocytes were centrifuged in a 5415C Eppendorf microfuge (2,000 rpm, 5 min). Total cell lysates were obtained using the M-PER (Mammalian Protein Extraction Reagent, ThermoFisher), according to the manufacturer's instructions. Aliquots of the protein extracts were stored at -80°C. Protein content was determined by Bradford (34).

Statistical Analyses

To examine differences between groups, unpaired Student's t tests (two-tailed) were used. To examine relationships between variables, bivariate Pearson's correlation analyses were performed, using GraphPad Prism version 8 software, which was used to construct all graphs. Principal Component Analyses (PCA) were generated using RStudio Version 1.1.463.

RESULTS

The Plasma of Individuals With Obesity Is Enriched in IgG Antibodies Specific for dsDNA, MDA, and Adipocyte-Derived Antigens

Plasma samples were isolated from individuals with obesity and from lean controls. Samples were tested for the presence of IgG antibodies specific for ds-DNA, MDA and adipocyte-derived antigens. **Figure 1** shows significantly higher amounts of IgG for the 3 different antigenic specificities in obese versus lean individuals.

We also measured IgM antibodies specific for the above autoantigens. Results show no significant differences in lean versus obese individuals for anti-ds-DNA IgM antibodies (0.84 ± 0.11 *vs.* 0.93 ± 0.13, p=0.60, n=6), for MDA IgM antibodies (1.39 ± 0.09 *vs.* 1.58 ± 0.08, p=0.15, n=6), and for IgM specific for adipocyte-derived antigens (1.26 ± 0.13 *vs.* 1.33 ± 0.09, p=0.06, n=18).

The Frequencies of DN B Cells Significantly Increase in the Blood of Obese Versus Lean Individuals

We have previously shown that DN B cells present in the blood and in the adipose tissue of individuals with obesity are responsible for the secretion of anti-adipocyte-specific IgG antibodies (15). Here, we tested the hypothesis that DN B cells were also associated with/responsible for the secretion of anti-dsDNA and anti-MDA IgG antibodies in the blood of obese individuals.

We therefore compared the frequencies of DN B cells in this cohort of obese and lean individuals. **Figure 2** (top) shows the major B cell subsets, gated on leukocytes (CD45+): naive (IgD+CD27-), IgM memory (IgD+CD27+), switched memory (swIg, IgD-CD27+) and DN (IgD-CD27-). Results in **Figure 2** (bottom) show the significant increase in the frequencies of DN B cells in obese versus lean individuals, confirming and extending to this cohort our previously published findings (10, 15). Results in **Figure 2** (bottom) also show the frequencies of the other B cell subsets. We observed a significant increase in the frequencies of naïve and a significant decrease in the frequencies of IgM memory B cells in obese versus lean individuals, whereas the frequencies of swIg were found not significantly different between the two groups. These results are slightly different from those we have previously published (10), likely because in this study we have included individuals that are older (27–55 years) than those in our previous study (20–40 years).

IgG Antibodies Specific for dsDNA, MDA, Adipocyte-Derived Antigens Are Positively Associated With Blood Frequencies of DN B Cells

As expected, IgG antibodies specific for the self-antigens in **Figure 1** were positively associated with blood frequencies of DN B cells in **Figure 2** (**Figure 3**).

DN B Cells Are Characterized by Higher Expression of IA Markers Associated With Autoimmunity

In order to characterize the phenotype of DN B cells present in the blood of individuals with obesity and of lean controls, we examined membrane expression of markers of IA, previously shown to be present on DN B cells from patients with autoimmunity. Briefly, we measured the following: CD21, the complement receptor for C3d (35); CD95, Fas ligand (36); CD11c, the Itgax integrin involved in antigen presentation to T cells (37); CD86 and HLADR, also involved in antigen presentation to T cells (38, 39); PD1, a marker of IA and of

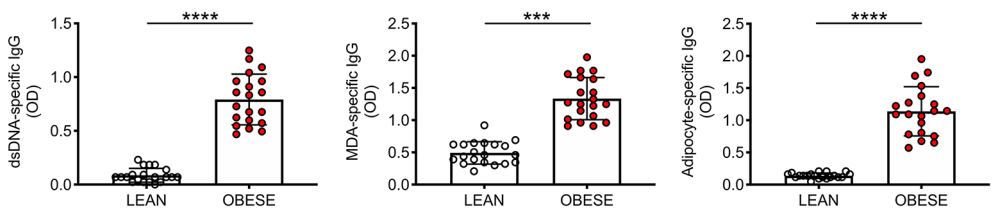

FIGURE 1 | The plasma of individuals with obesity is enriched in IgG antibodies specific for dsDNA, MDA and adipocyte-derived antigens. Plasma samples were isolated from individuals with obesity and from lean controls. Mean comparisons between groups were performed by Student's t test (two-tailed). ***p < 0.001, ****p < 0.0001.

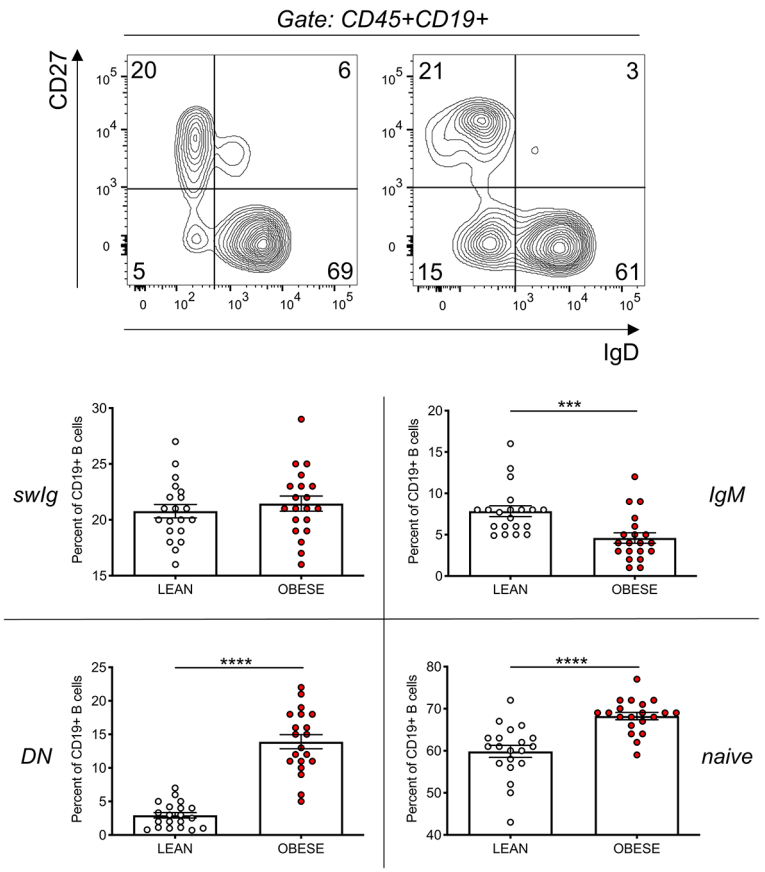

FIGURE 2 | The frequencies of DN B cells significantly increase in the blood of obese versus lean individuals. Top. Gating strategies and a representative dot plot from one lean and one obese individual. Bottom. Results show frequencies of the four B cell subsets. Mean comparisons between groups were performed by Student's t test (two-tailed). ***p < 0.001, ****p < 0.0001.

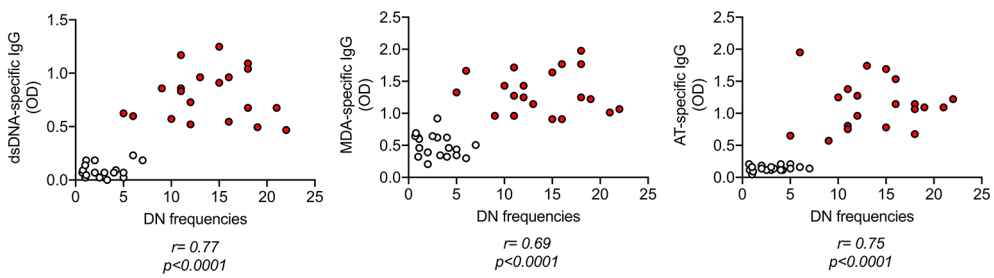

FIGURE 3 | IgG antibodies specific for dsDNA, MDA, adipocyte-derived antigens are positively associated with blood frequencies of DN B cells. IgG antibodies were measured in plasma as indicated in **Figure 1**. DN B cell frequencies were measured by flow cytometry as indicated in **Figure 2**. Correlation coefficients and p values are shown for each antibody specificity.

cell exhaustion (40). Results in **Figure 4A** show that DN B cells from individuals with obesity are characterized by lower levels of expression of CD21, and higher levels of expression of CD95, CD11c, CD86, HLADR, PD1, as compared to those from lean controls. These results are in agreement with previously published observations showing the association of the membrane phenotype CD21^{low}CD95+CD11c+CD86+HLADR+PD1+ with autoimmune B cell subsets, and clearly demonstrate that obesity induces the expansion of DN B cells characterized by this autoimmune phenotype. In the PCA

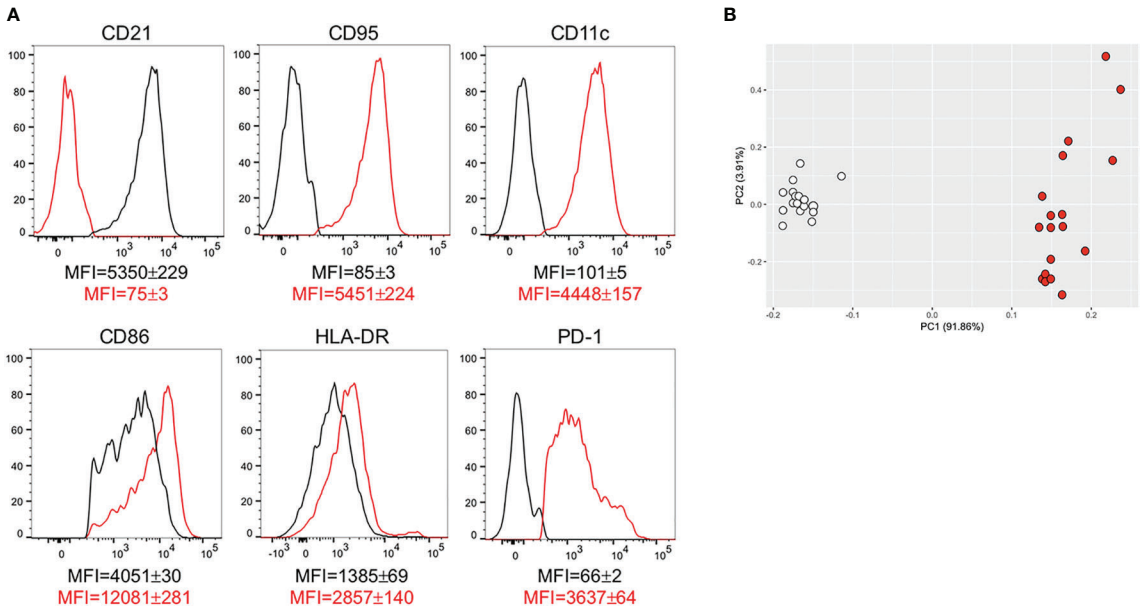

FIGURE 4 | DN B cells are characterized by higher expression of IA markers associated with autoimmunity. **(A)** Cells were stained to evaluate the expression of several markers of IA on DN B cells from individuals with obesity and from lean controls. Results show mean fluorescence intensity (MFI)± SE for each marker in DN B cells from lean (black line) and obese (red line) individuals (18 individuals/group). **(B)** PCA analysis with the axes showing the percentage of variation explained by PC1 and PC2. Each symbol indicates an individual. White symbols: lean individuals. Red symbols: obese individuals.

analysis in **Figure 4B** distinct clustering of DN B cells from the two groups of individuals are shown.

DN B Cells Are Also Characterized by Higher Expression of the Transcription Factor T-Bet Associated With Autoimmunity

Next, we evaluated if DN B cells with the membrane phenotype associated with autoimmunity were expressing not only the transcription factor T-bet, known to be involved in the secretion of anti–self-antibodies, but also the expression of transcription factors and enzymes crucial for CSR. Briefly, we measured RNA expression of T-bet (tbx21) and other transcription factors involved in CSR (E47, Pax-5), in germinal center reactions (bcl6), in plasma cell differentiation (prdm1, XBP1), as well as RNA expression of AID (aicda). Results in **Figure 5** show that tbx21, bcl6, aicda, prdm1 and XBP1 are all significantly up-regulated in unstimulated DN B cells from individuals with obesity as compared to lean controls. No differences were observed for E47 and Pax-5. These results show that DN B cells isolated from the blood of individuals with obesity, as compared to those isolated from lean controls, are not only already pre-activated, as indicated by their higher expression of IA markers, but also show spontaneous expression of the transcription factors associated with antibody secretion, including T-bet, associated with the secretion of IgG antibodies with anti–self–specificity.

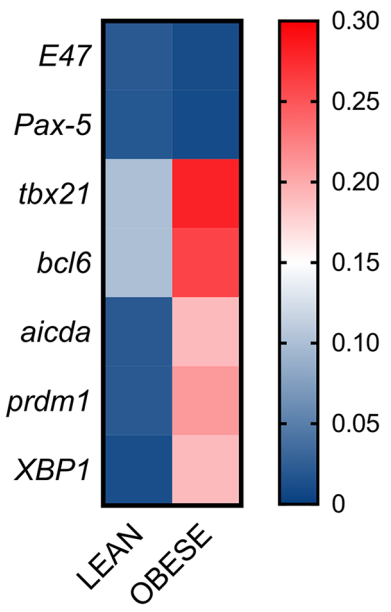

FIGURE 5 | DN B cells are characterized by higher expression of transcription factors associated with autoimmunity. DN B cells were sorted from the peripheral blood of individuals with obesity and of lean controls and left unstimulated. Total RNA was extracted to evaluate by qPCR the expression of transcription factors. Heatmap shows qPCR values ($2^{-\Delta\Delta Ct}$) of several transcription factors, normalized to GAPDH. Results show average qPCR values from 18 individuals/group.

The Removal of DN B Cells Significantly Reduces the Secretion of IgG Autoimmune Antibodies

We have previously shown that DN B cells sorted from the breast adipose tissue of obese female patients undergoing weight reduction surgeries secrete autoimmune IgG antibodies that are specific for adipocyte-derived antigens (15). These experiments have been possible because from surgery patients we get large pieces of discarded tissue and, also, because DN B cell frequencies in the adipose tissue reach up to 80% of the total B cell pool, a frequency never observed in the peripheral blood. To further confirm that DN B cells are responsible for the secretion of autoimmune IgG antibodies in the blood of individuals with obesity, we performed the following experiment. B cells, as well as B cells without DN B cells, isolated from the blood of individuals with obesity, were stimulated for 10 days with the B cell mitogen CpG. Stimulation is necessary to allow the stimulation/expansion of IgG secreting B cells. After stimulation, supernatants were collected and IgG autoimmune antibodies measured by ELISA. Results in **Figure 6** show that the removal of DN B cells from the pool of total B cells of obese individuals significantly decreased *in vitro* secretion of anti-dsDNA, anti-MDA and anti-adipocyte IgG specific antibodies.

DISCUSSION

The subset of DN B cells has been the focus of increasing interest in the last few years, as shown by a large number of dedicated publications. DN B cells expand in healthy aging, in autoimmune diseases, in chronic and acute infections. DN B cells also increase in the blood of individuals with obesity and reach significantly high frequencies in the obese subcutaneous adipose tissue, where they secrete large amounts of autoimmune antibodies with different specificities. As we have recently demonstrated, these specificities include adipocyte-derived products, mainly cell-associated proteins and nucleic acids, not known as autoantigens but released in large amounts in the obese adipose tissue under conditions associated with hypoxia and cell death (41). The finding that anti-dsDNA, anti-MDA and anti-adipocyte specific antibodies are increased in the plasma of healthy elderly individuals (15, 42) and obese individuals has suggested that obesity may accelerate age-associated B cell defects. Fat mass indeed increases with age in humans (43, 44) and this is associated with increased inflammaging (1), metabolic dysfunction (5, 45) and development of insulin resistance which also increases with age (46). Moreover, an age-associated increase in the ectopic deposit of triglycerides in several tissues (liver, muscle, heart, pancreas, kidney) (47–51) and in blood vessels (52) occurs, and this is associated with the development and/or progression of age-associated diseases.

Data herein clearly show that DN B cells from individuals with obesity express higher levels of membrane markers of IA associated with autoimmunity as compared to lean controls and are characterized by the phenotype CD21^{low}CD95+CD11c+CD86+HLADR+PD1+. They also spontaneously express higher RNA levels for transcription factors involved in the secretion of

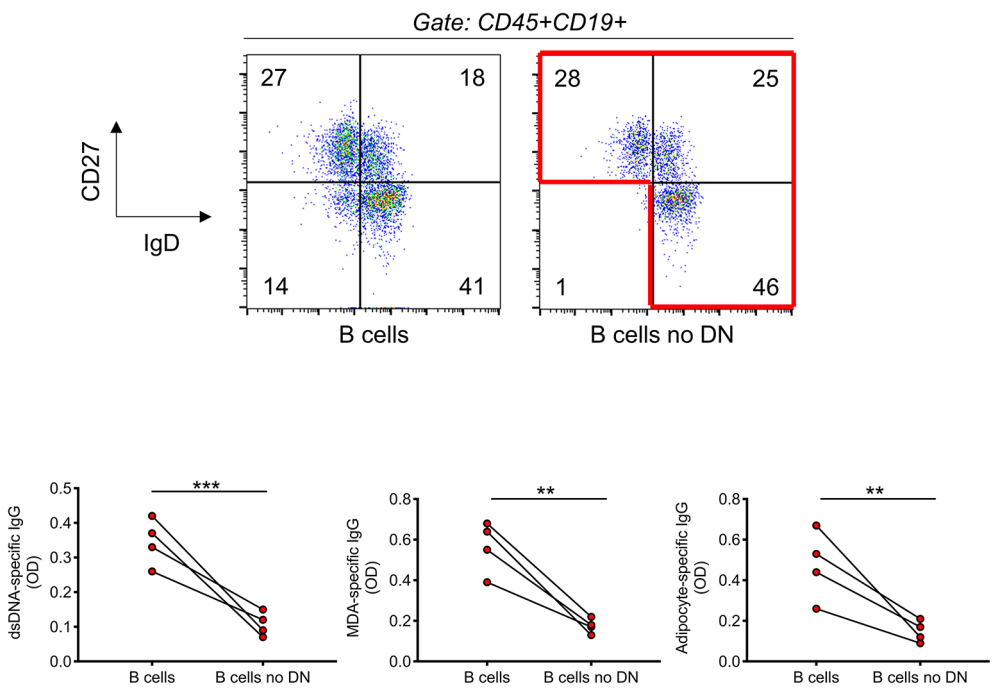

FIGURE 6 | The removal of DN B cells significantly reduces the secretion of IgG autoimmune antibodies. B cells were isolated with magnetic beads from the blood of four individuals with obesity. Top. Gating strategies to remove DN B cells. B cells were stained with anti-CD27 and anti-IgD antibodies. DN B cells were sorted out using a Sony SH800 cell sorter. Bottom. After stimulation of total B cells and total B cells without DN B cells for 10 days with CpG, supernatants were collected and analyzed for the presence of anti-dsDNA, anti-MDA, and anti-adipocyte IgG by ELISA. **p < 0.01, ***p < 0.001.

autoimmune antibodies (tbx21, prdm1, XBP1), suggesting that DN B cells from obese individuals are already pre-activated, a status leading to spontaneous secretion of autoimmune antibodies, as shown in autoimmune diseases (53), and in the obese adipose tissue at least for some specificities (33, 41). Because the IA phenotype of DN B cells from obese individuals is associated with increased energy demands, DN B cells engage in robust metabolic reprogramming to generate sufficient energy to fuel these demands and support autoantibody secretion (15).

Human DN B cells have many similarities with mouse splenic Age-associated B Cells (ABCs) (54, 55), identified as CD19+AA4.1-CD43-CD21-CD23- cells (54–56). DN B cells and ABCs originate from mature B cell subsets (naïve in humans, follicular B cells in mice) after *in vivo* or *in vitro* stimulation with the Toll-like receptors TLR7 or TLR9, alone or together with BCR cross-linking, demonstrating that BCR is also an active signaling system in these subsets. It has been shown that TLR agonists plus IL-21 and IFN-γ regulate T-bet expression, the transcription factor for the secretion of autoimmune antibodies (57), whereas TLR agonists plus IL-21 alone promote CD11c expression independently of T-bet (58). In agreement with the expression of T-bet, both human DN B cells and mouse ABCs secrete anti-ds-DNA (our results herein) or anti-chromatin (55) autoimmune IgG antibodies. Moreover, T-bet+ ABCs carry somatically mutated Ig, suggesting that they originate during T-dependent B cell responses (59). T-bet+ ABCs appear and persist indefinitely after influenza infection in mice (58, 59). These cells represent the spleen-resident population of memory B cells responsible for the secretion of HA stalk-specific IgG2c antibodies and of durable neutralizing antibodies (60). Previous results from Swain's group have also demonstrated that mouse ABCs are specific for a live influenza virus (A/PR8/34) and these influenza-specific ABCs differentiate into antibody-secreting cells, some of which home to the bone marrow and to the lungs where they persist for months, suggesting their role in providing significant protection (61). Human T-bet+ B cells also have also recently been shown to mediate influenza-specific humoral memory (60). Similar to mouse T-bet+ ABCs, they have an activated phenotype, they are spleen-resident and secrete HA-specific IgG1 antibodies recognizing H1 or H3 viral strains. IgG1 antibodies represent the equivalent of mouse IgG2c.

DN B cells are heterogeneous with two major subsets, DN1 and DN2. DN1 B cells are exclusively involved in follicular T-dependent antibody responses. DN2 B cells, conversely, represent the DN B cell subset that participates in extra-follicular B cell responses. DN1 B cells represent the major DN B cell subset in healthy individuals, whereas DN2 B cells increase in the blood of SARS-CoV-2-infected patients as compared to uninfected controls, suggesting a pathogenic role of DN2 B cells in COVID-19 patients (29). DN2 B cells also increase in the blood of SLE patients, as shown by the same group (62). In both cases, DN2 B cells are characterized by decreased expression of the chemokine receptor CXCR5, associated with follicular homing predisposition, and by a concomitant increased expression of CXCR3, a marker of homing to inflamed tissues. We haven't been able to identify DN1 and DN2 B cells in the blood and in the adipose tissue of individuals with obesity, likely because the individuals recruited in our studies are healthy, and either acute infection (COVID-19) or active disease (SLE) may be needed to allow the expansion of these extra-follicular B cells. We believe that the DN B cells in obese individuals are predominantly DN2, as they secrete autoimmune antibodies as observed in SLE patients.

In conclusion, our results confirm and extend our previous findings showing that frequencies of DN B cells increase in the blood of obese as compared to lean individuals and are positively correlated with the amounts of plasma autoimmune IgG antibodies. DN B cells are characterized by higher expression of IA markers and of the transcription factor T-bet, both associated with autoimmunity. When we removed DN B cells from the B cell pool we saw a significant decrease in the *in vitro* secretion of anti-self IgG antibodies. We believe that the results herein strongly support the role of DN B cells in the secretion of anti-self IgG antibodies in individuals with obesity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by institutional review board (IRB) protocol 20070481 and 20160542. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DF wrote the paper. AD, MR, and DF performed the experiments and acquired and analyzed data. DF and BB were involved in funding acquisition. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* (2000) 908:244–54. doi: 10.1111/j.1749-6632.2000.tb06651.x
2. Apovian CM, Gokce N. Obesity and cardiovascular disease. *Circulation* (2012) 125(9):1178–82. doi: 10.1161/CIRCULATIONAHA.111.022541
3. Casas R, Sacanella E, Estruch R. The immune protective effect of the Mediterranean diet against chronic low-grade inflammatory diseases. *Endocr Metab Immune Disord Drug Targets* (2014) 14(4):245–54. doi: 10.2174/1871530314666140922153350

4. Hass DJ, Brensinger CM, Lewis JD, Lichtenstein GR. The impact of increased body mass index on the clinical course of Crohn's disease. *Clin Gastroenterol Hepatol* (2006) 4(4):482–8. doi: 10.1016/j.cgh.2005.12.015
5. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* (2006) 444 (7121):860–7. doi: 10.1038/nature05485
6. Johnson AM, Olefsky JM. The origins and drivers of insulin resistance. *Cell* (2013) 152(4):673–84. doi: 10.1016/j.cell.2013.01.041
7. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* (2008) 371(9612):569–78. doi: 10.1016/S0140-6736(08)60269-X
8. Setty AR, Curhan G, Choi HK. Obesity, waist circumference, weight change, and the risk of psoriasis in women: Nurses' Health Study II. *Arch Intern Med* (2007) 167(15):1670–5. doi: 10.1001/archinte.167.15.1670
9. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest* (2006) 116(7):1793–801. doi: 10.1172/JCI29069
10. Frasca D, Ferracci F, Diaz A, Romero M, Lechner S, Blomberg BB. Obesity decreases B cell responses in young and elderly individuals. *Obesity (Silver Spring)* (2016) 24(3):615–25. doi: 10.1002/oby.21383
11. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* (2000) 102 (5):553–63. doi: 10.1016/s0092-8674(00)00078-7
12. Frasca D, Diaz A, Romero M, Landin AM, Phillips M, Lechner SC, et al. Intrinsic defects in B cell response to seasonal influenza vaccination in elderly humans. *Vaccine* (2010) 28(51):8077–84. doi: 10.1016/j.vaccine.2010.10.023 doi: S0264-410X(10)01492-1 [pii].
13. Frasca D, Diaz A, Romero M, Phillips M, Mendez NV, Landin AM, et al. Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. *Int Immunol* (2012) 24:175–82. doi: 10.1093/intimm/dxr123 doi: dxr123 [pii].
14. Khurana S, Frasca D, Blomberg B, Golding H. AID activity in B cells strongly correlates with polyclonal antibody affinity maturation in-vivo following pandemic 2009-H1N1 vaccination in humans. *PloS Pathog* (2012) 8(9): e1002920. doi: 10.1371/journal.ppat.1002920
15. Frasca D, Diaz A, Romero M, Thaller S, Blomberg BB. Metabolic requirements of human pro-inflammatory B cells in aging and obesity. *PloS One* (2019) 14(7):e0219545. doi: 10.1371/journal.pone.0219545
16. Colonna-Romano G, Bulati M, Aquino A, Pellicano M, Vitello S, Lio D, et al. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech Ageing Dev* (2009) 130(10):681–90. doi: 10.1016/j.mad.2009.08.003
17. Frasca D, Diaz A, Romero M, Blomberg BB. Human peripheral late/exhausted memory B cells express a senescent-associated secretory phenotype and preferentially utilize metabolic signaling pathways. *Exp Gerontol* (2017) 87 (Pt A):113–20. doi: 10.1016/j.exger.2016.12.001
18. Nevalainen T, Autio A, Kummola L, Salomaa T, Junttila I, Jylha M, et al. CD27- IgD- B cell memory subset associates with inflammation and frailty in elderly individuals but only in males. *Immun Ageing* (2019) 16:19. doi: 10.1186/s12979-019-0159-6
19. Adlowitz DG, Barnard J, Biear JN, Cistrone C, Owen T, Wang W, et al. Expansion of Activated Peripheral Blood Memory B Cells in Rheumatoid Arthritis, Impact of B Cell Depletion Therapy, and Biomarkers of Response. *PloS One* (2015) 10(6):e0128269. doi: 10.1371/journal.pone.0128269
20. Wang S, Wang J, Kumar V, Karnell JL, Naiman B, Gross PS, et al. IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c(hi) T-bet(+) B cells in SLE. *Nat Commun* (2018) 9(1):1758. doi: 10.1038/s41467-018-03750-7
21. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. *Clin Immunol* (2004) 113(2):161–71. doi: 10.1016/j.clim.2004.05.010
22. Claes N, Fraussen J, Vanheusden M, Hellings N, Stinissen P, Van Wijmeersch B, et al. Age-Associated B Cells with Proinflammatory Characteristics Are Expanded in a Proportion of Multiple Sclerosis Patients. *J Immunol* (2016) 197(12):4576–83. doi: 10.4049/jimmunol.1502448
23. Martorana A, Balistreri CR, Bulati M, Buffa S, Azzarello DM, Camarda C, et al. Double negative (CD19+IgG+IgD-CD27-) B lymphocytes: a new insight from telomerase in healthy elderly, in centenarian offspring and in Alzheimer's disease patients. *Immunol Lett* (2014) 162(1 Pt B):303–9. doi: 10.1016/j.imlet.2014.06.003
24. Saadoun D, Terrier B, Bannock J, Vazquez T, Massad C, Kang I, et al. Expansion of autoreactive unresponsive CD21-/low B cells in Sjogren's syndrome-associated lymphoproliferation. *Arthritis Rheum* (2013) 65 (4):1085–96. doi: 10.1002/art.37828
25. Golinski ML, Demeules M, Derambure C, Riou G, Maho-Vaillant M, Boyer O, et al. CD11c(+) B Cells Are Mainly Memory Cells, Precursors of Antibody Secreting Cells in Healthy Donors. *Front Immunol* (2020) 11:32. doi: 10.3389/fimmu.2020.00032
26. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med* (2008) 205 (8):1797–805. doi: 10.1084/jem.20072683
27. Chang LY, Li Y, Kaplan DE. Hepatitis C viraemia reversibly maintains subset of antigen-specific T-bet+ tissue-like memory B cells. *J Viral Hepat* (2017) 24 (5):389–96. doi: 10.1111/jvh.12659
28. Illingworth J, Butler NS, Roetynck S, Mwacharo J, Pierce SK, Bejon P, et al. Chronic exposure to Plasmodium falciparum is associated with phenotypic evidence of B and T cell exhaustion. *J Immunol* (2013) 190(3):1038–47. doi: 10.4049/jimmunol.1202438
29. Woodruff MC, Ramonell RP, Nguyen DC, Cashman KS, Saini AS, Haddad NS, et al. Extrafollicular B cell responses correlate with neutralizing antibodies and morbidity in COVID-19. *Nat Immunol* (2020) 21(12):1506–16. doi: 10.1038/s41590-020-00814-z
30. Wlodarczyk M, Nowicka G. Obesity, DNA Damage, and Development of Obesity-Related Diseases. *Int J Mol Sci* (2019) 20(5):1146–63. doi: 10.3390/ijms20051146
31. Sankhla M, Sharma TK, Mathur K, Rathor JS, Butolia V, Gadhok AK, et al. Relationship of oxidative stress with obesity and its role in obesity induced metabolic syndrome. *Clin Lab* (2012) 58(5-6):385–92. doi: 10.1172/JCI21625
32. Yesilbursa D, Serdar Z, Serdar A, Sarac M, Coskun S, Jale C. Lipid peroxides in obese patients and effects of weight loss with orlistat on lipid peroxides levels. *Int J Obes (Lond)* (2005) 29(1):142–5. doi: 10.1038/sj.ijo.0802794
33. Frasca D, Diaz A, Romero M, Thaller S, Blomberg BB. Secretion of autoimmune antibodies in the human subcutaneous adipose tissue. *PloS One* (2018) 13(5):e0197472. doi: 10.1371/journal.pone.0197472
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* (1976) 72:248–54. doi: 10.1006/abio.1976.9999
35. Cherukuri A, Cheng PC, Pierce SK. The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. *J Immunol* (2001) 167(1):163–72. doi: 10.4049/jimmunol.167.1.163
36. Jacobi AM, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, et al. Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95. *Arthritis Rheum* (2008) 58(6):1762–73. doi: 10.1002/art.23498
37. Rubtsov AV, Rubtsova K, Kappler JW, Jacobelli J, Friedman RS, Marrack P. CD11c-Expressing B Cells Are Located at the T Cell/B Cell Border in Spleen and Are Potent APCs. *J Immunol* (2015) 195(1):71–9. doi: 10.4049/jimmunol.1500055
38. June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. *Immunol Today* (1994) 15(7):321–31. doi: 10.1016/0167-5699(94)90080-9
39. Schwartz BD. HLA molecules: sentinels of the immune response. *Am J Respir Cell Mol Biol* (1991) 5(3):211–2. doi: 10.1165/ajrcmb/5.3.211
40. Thibult ML, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L, et al. PD-1 is a novel regulator of human B-cell activation. *Int Immunol* (2013) 25(2):129–37. doi: 10.1093/intimm/dxs098
41. Frasca D, Diaz A, Romero M, Garcia D, Jayram D, Thaller S, et al. Identification and Characterization of Adipose Tissue-Derived Human Antibodies With "Anti-self" Specificity. *Front Immunol* (2020) 11:392. doi: 10.3389/fimmu.2020.00392
42. Ruffatti A, Calligaro A, Del Ross T, Bertoli MT, Doria A, Rossi L, et al. Anti-double-stranded DNA antibodies in the healthy elderly: prevalence and characteristics. *J Clin Immunol* (1990) 10(6):300–3. doi: 10.1007/BF00917474
43. Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, et al. Fat tissue, aging, and cellular senescence. *Aging Cell* (2010) 9 (5):667–84. doi: 10.1111/j.1474-9726.2010.00608.x

44. Zamboni M, Rossi AP, Fantin F, Zamboni G, Chirumbolo S, Zoico E, et al. Adipose tissue, diet and aging. *Mech Ageing Dev* (2014) 136-137:129–37. doi: 10.1016/j.mad.2013.11.008
45. Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders. *Nature* (2017) 542(7640):177–85. doi: 10.1038/nature21363
46. Muller DC, Elahi D, Tobin JD, Andres R. The effect of age on insulin resistance and secretion: a review. *Semin Nephrol* (1996) 16(4):289–98.
47. Foster MC, Hwang SJ, Porter SA, Massaro JM, Hoffmann U, Fox CS. Fatty kidney, hypertension, and chronic kidney disease: the Framingham Heart Study. *Hypertension* (2011) 58(5):784–90. doi: 10.1161/HYPERTENSIONAHA.111.175315
48. Machann J, Thamer C, Schnoedt B, Stefan N, Stumvoll M, Haring HU, et al. Age and gender related effects on adipose tissue compartments of subjects with increased risk for type 2 diabetes: a whole body MRI/MRS study. *MAGMA* (2005) 18(3):128–37. doi: 10.1007/s10334-005-0104-x
49. Ryan AS, Nicklas BJ. Age-related changes in fat deposition in mid-thigh muscle in women: relationships with metabolic cardiovascular disease risk factors. *Int J Obes Relat Metab Disord* (1999) 23(2):126–32. doi: 10.1038/sj.ijo.0800777
50. Saisho Y, Butler AE, Meier JJ, Monchamp T, Allen-Auerbach M, Rizza RA, et al. Pancreas volumes in humans from birth to age one hundred taking into account sex, obesity, and presence of type-2 diabetes. *Clin Anat* (2007) 20(8):933–42. doi: 10.1002/ca.20543
51. Silaghi A, Piercecchi-Marti MD, Grino M, Leonetti G, Alessi MC, Clement K, et al. Epicardial adipose tissue extent: relationship with age, body fat distribution, and coronaropathy. *Obesity (Silver Spring)* (2008) 16(11):2424–30. doi: 10.1038/oby.2008.379
52. Robert L. Aging of the vascular-wall and atherosclerosis. *Exp Gerontol* (1999) 34(4):491–501. doi: 10.1016/s0531-5565(99)00030-3
53. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol Rev* (2005) 204:27–42. doi: 10.1111/j.0105-2896.2005.00239.x
54. Hao Y, O'Neill P, Naradikian MS, Scholz JL, Cancro MP. A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. *Blood* (2011) 118(5):1294–304. doi: 10.1182/blood-2011-01-330530
55. Rubtsov AV, Rubtsova K, Fischer A, Meehan RT, Gillis JZ, Kappler JW, et al. Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c(+) B-cell population is important for the development of autoimmunity. *Blood* (2011) 118(5):1305–15. doi: 10.1182/blood-2011-01-331462
56. Frasca D, Diaz A, Romero M, Vazquez T, Blomberg BB. Obesity induces pro-inflammatory B cells and impairs B cell function in old mice. *Mech Ageing Dev* (2017) 162:91–9. doi: 10.1016/j.mad.2017.01.004
57. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A* (2002) 99(8):5545–50. doi: 10.1073/pnas.082114899
58. Naradikian MS, Myles A, Beiting DP, Roberts KJ, Dawson L, Herati RS, et al. Cutting Edge: IL-4, IL-21, and IFN-gamma Interact To Govern T-bet and CD11c Expression in TLR-Activated B Cells. *J Immunol* (2016) 197(4):1023–8. doi: 10.4049/jimmunol.1600522
59. Russell Knode LM, Naradikian MS, Myles A, Scholz JL, Hao Y, Liu D, et al. Age-Associated B Cells Express a Diverse Repertoire of VH and Vkappa Genes with Somatic Hypermutation. *J Immunol* (2017) 198(5):1921–7. doi: 10.4049/jimmunol.1601106
60. Johnson JL, Rosenthal RL, Knox JJ, Myles A, Naradikian MS, Madej J, et al. The Transcription Factor T-bet Resolves Memory B Cell Subsets with Distinct Tissue Distributions and Antibody Specificities in Mice and Humans. *Immunity* (2020) 52(5):842–55 e6. doi: 10.1016/j.immuni.2020.03.020
61. Swain SL, Kugler-Umana O, Kuang Y, Zhang W. The properties of the unique age-associated B cell subset reveal a shift in strategy of immune response with age. *Cell Immunol* (2017) 321:52–60. doi: 10.1016/j.cellimm.2017.05.009
62. Jenks SA, Cashman KS, Zumaquero E, Marigorta UM, Patel AV, Wang X, et al. Distinct Effector B Cells Induced by Unregulated Toll-like Receptor 7 Contribute to Pathogenic Responses in Systemic Lupus Erythematosus. *Immunity* (2018) 49(4):725–39 e6. doi: 10.1016/j.immuni.2018.08.015

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Marginal Zone B Cells Assist With Neutrophil Accumulation to Fight Against Systemic *Staphylococcus aureus* Infection

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In addition to regulating immune responses by producing antibodies that confer humoral immunity, B cells can also affect these responses by producing cytokines. How B cells participate in the clearance of pathogenic infections *via* functions other than the production of pathogen-specific antibodies is still largely unknown. Marginal zone (MZ) B cells can quickly respond to bacterial invasion by providing the initial round of antibodies. After a bloodborne bacterial infection, neutrophils promptly migrate to the MZ. However, the mechanisms regulating neutrophil accumulation in the MZ during the initial phase of infection also remain obscure. Here, we found that MZ B cell-deficient mice are more susceptible to systemic *Staphylococcus aureus* (*S. aureus*) infection compared with wildtype mice. The expression levels of interleukin (IL)-6 and CXCL1/CXCL2 in MZ B cells increased significantly in mice at 3–4 h after infection with *S. aureus*, then decreased at 24 h post-infection. After systemic *S. aureus* infection, splenic neutrophils express increased CXCR2 levels. Our results from confocal microscopy imaging of thick-section staining demonstrate that neutrophils in wildtype mice form cell clusters and are in close contact with MZ B cells at 3 h post-infection. This neutrophil cluster formation shortly after infection was diminished in both MZ B cell-deficient mice and IL-6-deficient mice. Blocking the action of CXCL1/CXCL2 by injecting anti-CXCL1 and anti-CXCL2 antibodies 1 h before *S. aureus* infection significantly suppressed the recruitment of neutrophils to the MZ at 3 h post-infection. Compared with peptidoglycan stimulation alone, peptidoglycan stimulation with neutrophil co-culture further enhanced MZ B-cell activation and differentiation. Using a Förster resonance energy transfer by fluorescence lifetime imaging (FLIM-FRET) analysis, we observed evidence of a direct interaction between neutrophils and MZ B cells after peptidoglycan stimulation. Furthermore, neutrophil depletion in mice resulted in a reduced production of *S. aureus*-specific immunoglobulin (Ig)M at 24 h post-infection. Together, our results demonstrate that MZ B cells regulate the rapid neutrophil swarming into the spleen during the early phase of

systemic *S. aureus* infection. Interaction with neutrophils assists MZ B cells with their differentiation into IgM-secreting cells and contributes to the clearance of systemic bacterial infections.

Keywords: marginal zone B cell, IL-6, neutrophil, *Staphylococcus aureus*, FRET

INTRODUCTION

There is a growing body of evidence demonstrating that not only are B cells capable of producing antibodies but these cells also have a regulatory role in immune responses *via* cytokine production (1). B cells develop in the bone marrow, after which immature B cells travel to the secondary lymphoid tissues to further mature into transitional B cells (2). Transcription factor recombination signal binding protein-J (RBP-J) regulates downstream gene expression activated by Notch receptors. Notch–RBP-J signaling determines the development of mature B cells in the spleen into follicular B cells or marginal zone (MZ) B cells (3). MZ B cells are located outside the marginal sinus at the interface between the white pulp and red pulp in the spleen. Compared with follicular B cells, MZ B cells are in a preactivated state and thus respond faster to foreign pathogens (4–7). They provide timely defense in the initial phase of bloodborne microbial infection by detecting pathogens through toll-like receptors and B-cell receptors (8). MZ B cells integrate the roles of sensing pathogens and effector cells during T cell-independent immune responses, then promptly differentiate into plasma cells that secrete protective antibodies. MZ B cells can also participate in T cell-dependent immune responses by capturing bloodborne pathogens and transferring them to the follicular area of the spleen. These cells can rapidly produce IgM antibodies following appropriate stimulation and subsequently differentiate into short-lived plasma cells that aid in early immune responses, thus filling the protective gap between the innate and adaptive immune responses (9–11).

Interleukin (IL)-6 is the main inflammatory cytokine released by B cells during the initial stage of infection (12, 13). Studies have shown that IL-6 has both proinflammatory and anti-inflammatory properties. IL-6 binds to the membrane-bound receptor IL-6R, then forms a signaling complex *via* a glycoprotein 130 (gp130) homodimer to stimulate intracellular signaling pathways (14–16). This critical regulator of innate immunity promotes the transition of leukocyte recruitment during acute inflammation from neutrophils to monocytes *via* inducing the expression of chemokines, such as MCP-1 and CXCL8, in endothelial cells (17). IL-6 has also been shown to play an anti-inflammatory role through suppressing neutrophil recruitment during acute inflammatory responses (18). Whether IL-6 released by B cells participates in the regulation of neutrophil recruitment into the MZ during the early phase of bloodborne pathogen infection remains to be determined.

Neutrophils are generally short-lived, with a circulation half-life of 6–8 h (19). They form the main innate immune cell population that can quickly eliminate pathogenic invasion. Once neutrophils are recruited to the site of infection through the leukocyte adhesion cascade, these cells may function for 1–2 days in the tissues before undergoing apoptosis (20, 21). However, there are many factors capable of prolonging neutrophil survival during inflammatory responses, such as granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-α (TNFα), interferon γ, IL-6, and bacterial or fungal products (22, 23). Neutrophils show different phenotypes and functional responses after being primed by microbes (24). Several studies have indicated that certain neutrophils colonize in the red pulp region of the spleen in a noninflammatory state. When infection occurs, they quickly migrate to the infection site, together with circulating neutrophils, and release active molecules to control microbial infection. These splenic neutrophils provide critical help by releasing cytokines to promote antibody production by MZ B cells and immunoglobulin class switching (25–27). The infiltration of neutrophils into inflamed and infected tissues is initiated by a small number of neutrophils. They respond to the initial danger signals by secreting proinflammatory mediators and forming small clusters. Within a few minutes, the accumulation of a large number of neutrophils and the formation of neutrophil clusters then promote their coordinated effector functions in subsequent innate immune responses (28).

Neutrophils can interact with other cell types, including dendritic cells, macrophages, B cells, and T cells, *via* various receptors, such as chemoattractant receptors, fragment crystallizable (Fc)-receptors, cytokine receptors, Toll-like receptors, and C-type lectin receptors (29–31). Despite their short lifespan, neutrophils play a vital role in host defense against bacterial infection and regulate adaptive immunity (32). However, the details of the interaction between MZ B cells and neutrophils during the response to bloodborne microorganism infection remain largely unknown. *Staphylococcus aureus* is a Gram-positive human pathogen that is a cause of serious infections within communities and hospitals. Systemic *S. aureus* infections can cause severe disease manifestations, such as sepsis (33). Here, we investigate the regulatory loop between MZ B cells and neutrophils during acute systemic infection with *S. aureus*.

MATERIALS AND METHODS

Mice

All mice used in this study had a C57BL/6 genetic background. Wildtype (WT) C57BL/6 mice were purchased from BioLASCO and National Laboratory Animal Center in Taiwan (Taipei, Taiwan). The RBP-$J^{f/f}$ × CD19-Cre^{+} conditional knockout (RBP-J CKO) mice were kindly provided by Dr. Tasuku Honjo (Institute for Advanced Study, Kyoto University, Japan). The LysM-eGFP mice expressing enhanced green fluorescent protein (eGFP) in granulocytes were provided by Dr. Ellen Robey (UC

Berkeley, US) (34). The IL-6 knockout (IL-6 KO) mice were purchased from The Jackson Laboratory. All mice were bred and kept under specific pathogen-free conditions in Academia Sinica Animal Care Facility before infection with *S. aureus* in P2/P3 animal facility. Animal experimental procedures and the use of the animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Academia Sinica.

Flow Cytometry Analysis

Single-cell suspensions of splenocytes were stained with the following antibodies: anti-CD138-Brilliant Violet 421 monoclonal antibody (mAb; clone 281-2, Biolegend), anti-Ly6C-FITC mAb (clone HKL4, Biolegend), anti-CD21/35-APC mAb (clone 7E9, Biolegend), anti-CD23-PEcy7 mAb (clone B3B4, Biolegend), anti-B220-APCcy7 mAb (clone RA3-6B2, Biolegend), anti-Ly6G-FITC mAb (clone 1A8, Biolegend), anti-CD11b-PE mAb (clone M1/70, Biolegend), anti-CD69 mAb (clone H1.2F3, BD Biosciences), and anti-CD86 mAb (clone GL1, BD Biosciences). The applied staining methods adhered to the recommendations in "Guidelines for the use of flow cytometry and cell sorting in immunological studies" (35). Cells were fixed and permeabilized using Foxp3/Transcription Factor Fixation/Permeabilization Kit (Invitrogen; ThermoFisher REF 00-5523-00), and then stained with anti-IL-6 mAb (clone MP5-20F3, Biolegend) with anti-IL-6 APC mAb (clone MP5-20F3, Biolegend), anti-CD21/35-FITC mAb (clone 7E9, Biolegend), and anti-CD23-Brilliant Violet 421 mAb (clone B3B4, Biolegend) for detecting intracellular IL-6. Neutrophils ($Ly6G^{hi}CD11b^{hi}$), MZ B cells ($B220^{+}CD21^{hi}CD23^{lo}$), and plasma cells ($CD138^{+}$ or $CD138^{+}Ly6C^{+}$) were analyzed by using a BD FACSCanto II flow cytometer. In the co-culture experiments, mouse spleen MZ B cells ($B220^{+}CD21^{hi}CD23^{lo}$) were sorted by a BD FACSAria sorter. Flow cytometric data were analyzed by BD FlowJo software.

S. aureus Infection and Preparation of *S. aureus* Lysates

Eight- to ten-week-old mice were intravenously injected with $2.5–5 \times 10^6$ colony-forming units (CFU) of *S. aureus* (regular strain ATCC25923), after which the mouse survival and bodyweight changes were monitored daily for up to 10 days. In some experiments, mice were intraperitoneally administered 400 μg of anti-Ly6G antibody (clone 1A8, BioXCell) to deplete neutrophils or 400 μg of isotype control antibody (clone 2A3, BioXCell) as a control at 24 h before *S. aureus* infection. Four hours later, MZ B cells were isolated by using a FACSAria flow cytometer (BD) to perform the subsequent analysis. *S. aureus* lysates were prepared in the B-PER Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Briefly, *S. aureus* was grown overnight in trypticase soy broth, killed by treatment with 3% formalin for 30 min, washed twice with phosphate-buffered saline (PBS), and stored at −80°C before processing. Cell pellets were suspended in 4 ml of B-PER Bacterial Protein Extraction Reagent per gram of wet mass. DNase I (300 μg/ml, BD) was added into a solution containing an EDTA-free protease inhibitor cocktail (Roche) and combined with the bacterial mixture, which was then incubated for 15 min at room temperature on a shaking platform. Bacterial mixtures were subsequently sonicated on ice 10 times for 40 s each, with a 40-s interval between each sonication, by using a Q700 sonicator (QSonica). The resulting crude extracts were centrifuged at 10,000 ×*g* for 20 min at 4 °C, followed by the removal of cell debris.

Measurement of *S. aureus*-Specific IgM by Enzyme-Linked Immunosorbent Assay (ELISA)

Mice were intravenously injected with *S. aureus* (2.5×10^6 CFU) or left untreated, then sacrificed at the indicated times. The splenic tissue fluids in 1 ml of RPMI medium were harvested by collecting the supernatants resulting from centrifugation, and they were stored at −80°C before processing. A 96-well ELISA plate was precoated overnight with *S. aureus* lysate (70 μg/well), and the splenic tissue fluids were then subjected to ELISA analysis, performed in accordance with the manufacturer's instructions, to determine the post-infection levels of *S. aureus*-specific IgM in the spleen. The absorbance was detected at 450 nm by a microplate reader (SpectraMax M2).

Neutrophil Isolation

Splenocytes harvested from mice infected with *S. aureus* (2.5×10^6 CFU) were overlaid on four-layer Percoll gradients (45%-55%-62%-81%) (GE Healthcare). Neutrophils were collected, as previously described, from the interface of the 62%-81% layers after centrifugation at 3,000 ×*g* for 30 min without the use of a brake and were washed with Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (36, 37). A highly pure neutrophil population (90%–95% $Ly6G^{hi}CD11b^{hi}$), as assessed by flow cytometry, was isolated by using negative selection beads (Stemcell Technology) in accordance with the manufacturer's instructions.

Microarray Analysis

FACS-sorted MZ B cells ($B220^{+}CD21^{hi}CD23^{lo}$) and neutrophils isolated by negative selection beads (Stemcell Technology) were purified from mice before and 4 h after *S. aureus* (2.5×10^6 CFU) infection. RNA was extracted by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The isolated RNA (100 ng) combined from MZ B cells or neutrophils from multiple mice was subjected to a cDNA microarray analysis using a Clarion™ D Array, mouse (Affymetrix) in accordance with the manufacturer's instructions. The array data were acquired using a GeneChip Scanner 3000 (Affymetrix) and analyzed by GeneSpring. The raw data were deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE157176. Differentially expressed genes in MZ B cells or neutrophils, with an upregulation or downregulation of ≥1.5-fold from 0 h to 4 h post-infection are shown.

Real-Time PCR Analysis

An RNeasy Plus Mini Kit (Qiagen) was used to extract the total RNA from sorted MZ B cells or neutrophils isolated by negative

selection beads (Stemcell Technology) taken from mice at 0, 4, or 24 h post-infection with *S. aureus*, and cDNA was generated from the resulting RNA by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions. The resulting cDNA (10 ng) and gene-specific primer sets were used with the QuantStudio 5 Real-Time PCR System (Applied Biosystems) to perform a qPCR analysis. The primer pairs for SYBR green detection are listed below:

Cxcl1: 5'-GCAGACCATGGCTGGGATT-3' and 5'-TGTCA GAAGCCAGCGTTCAC-3',

Cxcl2: 5'-ACTGCGCCCAGACAGAAGTC-3' and 5'-CAGTTA GCCTTGCCTTTGTTCAG-3',

Cxcr1: 5'-CCATTCCGTTCTGGTACAGTCTG-3' and 5'-GTAGCAGACCAGCATAGTGAGC-3',

Cxcr2: 5'-CACTATTCTGCCAGATGCTGTCC-3' and 5'-ACAA GGCTCAGCAGAGTCACCA-3',

Il-6: 5'-TTCCATCCAGTTGCCTTCTTGG-3' and 5'-TTCTCA TTTCCACGATTTCCCAG-3',

Il-10: 5'-GCTCTTACTGACTGGCATGAGGAT-3' and 5'-GCTGGTCCTTTGTTTGAAAGAAAG-3', *Tnf*: 5'-GACCC TCACACTCAGATCATCTTCT-3' and 5'-CCTCCA CTTGGTGGTTTGCT-3'

Relative levels of mRNA were normalized to Actin expression in each sample. *Actin* are 5'-CATTGCTGACAGGATGCAGAAGG-3' and 5'-TGCTGGAAGGTGGACAGTGAGG-3'.

Cytokine Measurement

Uninfected mice or mice infected with *S. aureus* (2.5×10^6 CFU) were sacrificed at the indicated times. The splenic tissue fluids were then collected and used in an ELISA analysis to detect the levels of mouse IL-6 (Thermo Fisher Scientific). The absorbance at 450 nm was detected by using a microplate reader (SpectraMax M2). In the *in vitro* experiments, MZ B cells (1×10^6 cells) were sorted by the BD FACSAria system, seeded in 96-well plates precoated with 3% agarose gel in culture medium, then stimulated with 2 μg/ml peptidoglycan (PGN; InvivoGen) for 24 h. In co-culture experiments, neutrophils (1×10^6 cells/well) were seeded in 96-well plates precoated with 3% agarose, after which the sorted MZ B cells (1×10^6 cells/well) were added, and the resulting cultures were incubated for 24 h with or without PGN (2 μg/ml) or heat-killed *S. aureus* (MOI=10) stimulation. The preparation of heat-killed *S. aureus* was performed by incubating bacteria at 80°C for 30 min. The levels of proinflammatory cytokines in the culture supernatants were measured by using Cytometric Bead Array (CBA) Kits (BD Biosciences).

Confocal Microscopy Analysis of Thick Tissue Sections

In immunostaining experiments, mice were intravenously injected with *S. aureus* (2.5×10^6 CFU) and then sacrificed at the indicated times. In some experiments, mice were intravenously injected with 50 μg each of anti-CXCL1- and CXCL2-neutralizing antibodies (MAB453 and MAB452, R&D Systems) or equal amounts of IgG isotype control antibody (R&D Systems) 1 h before *S. aureus* infection. The spleens were harvested, fixed overnight in freshly prepared 4% paraformaldehyde in PBS, and washed three times for 10 min/wash with PBS. The spleens were then embedded in 4% agarose before being cut into 200-μm sections by using a vibrating microtome (Vibratome) (38, 39). The thick spleen sections were treated with freshly prepared sodium borohydride in PBS (1 mg/ml) three times for 10 min/wash to help reduce the background staining (40), and finally washed with PBS. The sections were then transferred into a permeabilization solution containing 1% Triton X-100 and 2% Tween 20 and incubated overnight at 4°C. Nonspecific binding was reduced by incubating the sections overnight in a blocking solution consisting of 10% normal goat serum in PBS at 4°C. The sections were washed three times with PBS for 10 min/wash at room temperature, then incubated for 2 days at 4°C with the following antibodies: anti-CD45R B220 (1:50 dilution, clone RA3-6B2, Violet Fluor 450, Merck), anti-mouse CD1d-PE (1:50 dilution, clone 1B1, Biolegend), and anti-mouse Ly6G-Alexa 647 (1:50 dilution, clone 1A8, Biolegend) antibodies. The sections were finally washed overnight with PBS in a rotating platform at 4°C, then mounted in Fluormount G (Southern Biotechnology Associates). The images were acquired by the Leica SP8 confocal microscope. Three spleens per experimental group at each timepoint were analyzed.

Förster Resonance Energy Transfer by Fluorescence Lifetime Imaging (FLIM-FRET) Analysis

The splenic neutrophils isolated from LysM-eGFP mice using negative selection beads (Stemcell Technology) were seeded in precoated dishes containing 3% agarose for 1 h, then co-cultured for 3 h at a 1:1 ratio with MZ B cells that had been sorted using an MZ and FO B Cell Isolation Kit (Miltenyi Biotec) under stimulation with PGN (2 μg/ml). The cells were then harvested and fixed in 4% paraformaldehyde, washed three times with PBS for 10 min/wash, blocked with 1% normal goat serum, and labeled with anti-CD19 primary antibody (1:100 dilution, Abcam) followed by Alexa Fluor 546-labeled secondary antibody (1:100 dilution, Biolegend) to detect MZ B cells. The FRET pairs are eGFP (serving as the donor fluorophore) and Alexa Fluor 546 (serving as the acceptor fluorophore). The Leica SP8 FALCON system was used to perform FLIM-FRET detection in the 488 nm argon laser channel for measuring the change in the fluorescence lifetime of the donor molecule.

Statistical Analysis

The statistical analyses in this study were primarily conducted by using GraphPad Prism 8 software (GraphPad Software, San Diego USA); p-values of <0.05 were considered statistically significant. Presented data are shown as the mean ± standard error of the mean (SEM). Unpaired Student's t-tests were used for comparing the differences between two groups. Comparisons between multiple groups were performed using a one-way

ANOVA, followed by Dunnetts' honestly significant difference *post hoc* test. The differences in mouse survival curves between the two groups were analyzed by a log-rank test.

RESULTS

Mice Lacking MZ B Cells Are More Susceptible to *S. aureus* Infection

Previous reports indicate that Notch–RBP-J signaling regulates the lineage commitment of MZ B cells but does not affect B1 cells or other lineage cells. The absence of RBP-J leads to the loss of MZ B cells but does not cause defects in B-cell homeostasis, differentiation, or activation (3). Here, we used RBP-J CKO mice that lacked MZ B cells (**Figure 1A**) to study the regulatory role of MZ B cells during infection with the common pathogenic bacteria *S. aureus*. We found that the survival rate after systemic infection with *S. aureus* of RBP-J CKO mice was significantly lower than that of littermate WT mice (**Figure 1B**). Similarly, the bodyweight loss after systemic *S. aureus* infection was more severe in RBP-J CKO mice as compared with that in WT mice (**Figure 1C**). These results indicate that mice lacking MZ B cells are more susceptible to systemic bacterial infections. We noticed that the differences in bodyweight loss between WT and RBP-J CKO mice occurred within 1–2 days after infection, so we wondered whether certain innate immune responses may work together with MZ B cells to clear the bloodborne bacterial infection.

We also checked the amounts of *S. aureus*-specific antibody after systemic infection in WT and RBP-J CKO mice. IgM is known to provide the first line of humoral immunity defense against pathogens and plays an important role during microbial infection (41). Indeed, we found that *S. aureus*-specific IgM production in the spleen increased significantly at 48 h after systemic infection (**Figure 1D**), which is linked with the increases in the percentage of plasma cells in the spleen at 24 h post-infection in WT mice (**Figure 1E**). In contrast, the amounts of *S. aureus*-specific IgM and the frequency of plasma cells produced by RBP-J CKO mice at 12, 24, and 48 h after systemic *S. aureus* infection were significantly lower (**Figures 1D, E**). Although the frequency of plasma cells decreased at 48 h post-infection in WT mice, these mice still had significantly higher percentages of splenic plasma cells compared with RBP-J CKO mice (**Figure 1E** and **Supplementary Figure 1A**). We also compared the frequency of plasma cells by using another more specific marker, $CD138^{+}Ly6C^{+}$ (42), at 24 h after *S. aureus* infection. Both approaches showed that the frequency of plasma cells in WT mice was significantly higher than that in RBP-J CKO mice at 24 h after infection (**Figure 1F** and **Supplementary Figure 1B**). These results suggest that MZ B cells contribute to the production of *S. aureus*-specific IgM in the early phase of infection and that the reduced levels of *S. aureus*-specific antibody are associated with the reduced survival of RBP-J CKO mice.

Cytokine IL-6 and Chemokine CXCL2 Were Upregulated in MZ B Cells After *S. aureus* Infection

B cells can produce a variety of cytokines/chemokines, such as IL-2, IL-4, TNFα, IL-6, IL-10, CXCL1, CXCL10, and CXCL13, in response to stimuli (43, 44). We hypothesized that MZ B cells may produce some cytokines/chemokines to regulate the recruitment of innate immune cells in systemic pathogen clearance, and thus sought to identify changes in the expression of cytokines/chemokines

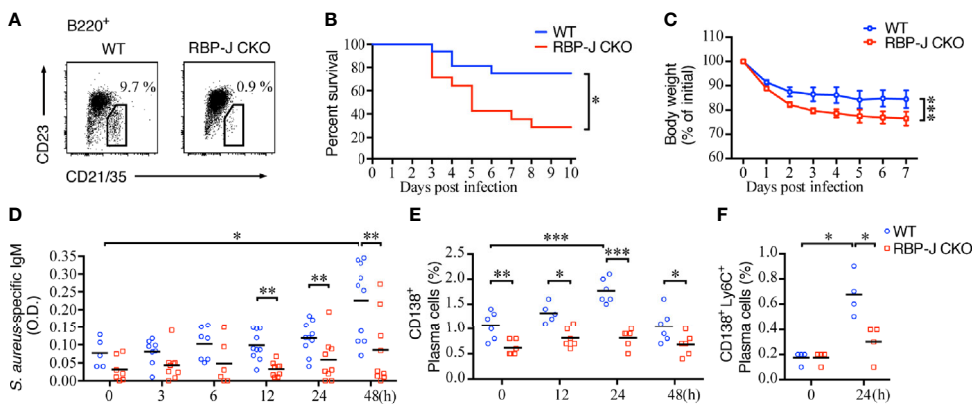

FIGURE 1 | Mortality and *S. aureus*-specific IgM production in mice lacking MZ B cells. **(A)** Splenocytes of RBP-$J^{f/f}$ × $CD19Cre^{+}$ (RBP-J CKO) or WT mice were stained with antibodies against B220, CD21/35, and CD23. MZ B cells were defined as $B220^{+}CD21/35^{hi}CD23^{lo}$ by flow cytometry. The numbers denote the percentage of MZ B cells in the B220 gate. **(B)** The survival rates of RBP-J CKO and WT mice were recorded daily after the mice were infected with 5 × 10^{6} CFU of *S. aureus*. The survival rate differences were analyzed by a log-rank (Mantel-Cox) test. **(C)** The bodyweight loss of mice after they were infected with 2.5 × 10^{6} CFU of *S. aureus*. The statistical analysis was conducted by performing an unpaired *t*-test (n = 7). **(D)** ELISA analysis using splenic tissue homogenates to measure the levels of *S. aureus*-specific IgM in RBP-J CKO and WT mice at various days after infection with 2.5 × 10^{6} CFU of *S. aureus*. The statistical analysis was conducted by performing an unpaired *t*-test (n = 5–10). **(E)** Differences in the percentages of $CD138^{+}$ plasma cells in RBP-J CKO and WT mice at the indicated timepoints after *S. aureus* infection were compared. **(F)** Differences in the percentage of $CD138^{+}Ly6C^{+}$ plasma cells in RBP-J CKO and WT mice at 24 h after *S. aureus* infection. The statistical analysis was conducted by performing an unpaired t-test (n = 4). Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

in MZ B cells after systemic *S. aureus* infection. Towards this end, sorted MZ B cells from uninfected mice or mice infected 4 h previously with *S. aureus* were subjected to cDNA microarray analyses. Eleven cytokine genes and thirteen chemokine genes in MZ B cells were found to be differentially upregulated or downregulated by at least 1.5 folds after infection (**Figure 2A** and **Supplementary Data Sheet 1**). Our RT-qPCR results confirmed that the *Cxcl1* and *Cxcl2* mRNA levels were increased at 4 h post-infection but were decreased at 24 h post-infection (**Figure 2B**). In addition, *Il6, Il10*, and *Tnfα* mRNA levels were also increased at 4 h post-infection, then reduced at 24 h post-systemic *S. aureus* infection (**Figure 2C**).

To validate whether the transcriptionally upregulated cytokines/chemokines were produced by MZ B cells after bacterial infection, we performed *in vitro* culture experiments using MZ B cells stimulated for 24 h with *S. aureus* peptidoglycan (PGN), which is a major component of the bacterial cell wall that contributes to the maintenance of mechanical strength and cell integrity (45).

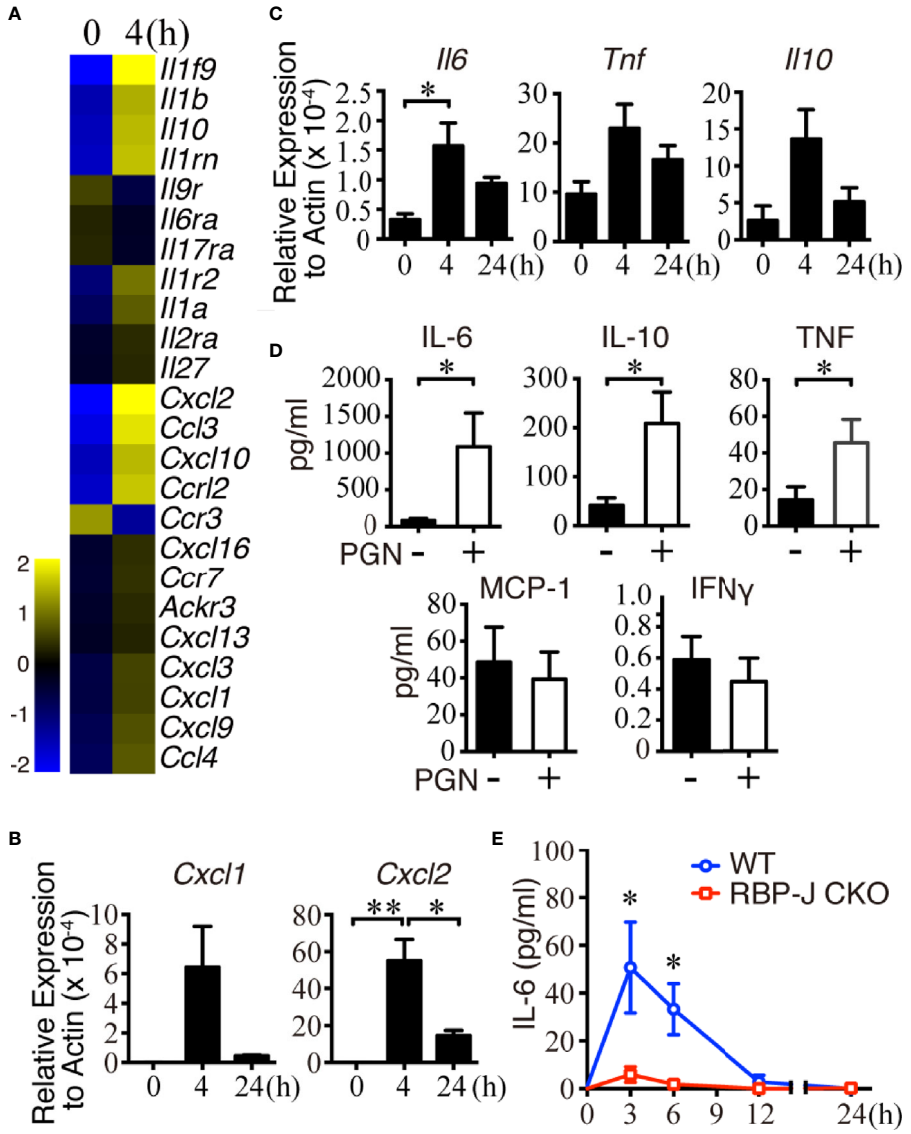

FIGURE 2 | Changes of cytokine and chemokine expression in MZ B cells in the early stage of *S. aureus* infection. **(A)** Changes in the expression of chemokine- and cytokine-related genes in MZ B cells isolated from mice after infection with *S. aureus* (2.5×10^6 CFU) by iv injection, as revealed by a cDNA microarray analysis. Heatmap showing the expression of several chemokine- and cytokine-related genes in MZ B cells. **(B)** RT-qPCR analysis of the *Cxcl1/Cxcl2* mRNA levels in MZ B cells isolated 0, 4, or 24 h after *S. aureus* infection. Results were normalized to the actin mRNA levels. **(C)** RT-qPCR analysis of the *Il6, Il10*, and *Tnf* mRNA levels in MZ B cells isolated 0, 4, or 24 h after *S. aureus* infection. Results were normalized to the actin mRNA levels. **(D)** Cytokines produced by purified MZ B cells treated with or without PGN (2 μg/ml) for 24 h were determined by a Cytometric Bead Array. **(E)** ELISA showing the levels of IL-6 in the splenic tissue homogenates of RBP-J CKO and WT mice at 0, 3, 6, 12, and 24 h after *S. aureus* infection (2.5×10^6 CFU). Statistical analysis was conducted by performing an unpaired *t*-test **(C, E)** and one-way ANOVA **(B, D)**. Data are presented as the mean ± SEM (n = 3 in B, n = 6–7 in C, n = 3–6 in D, and n = 6 in E). $^*p < 0.05$ and $^{**}p < 0.01$.

Cytometric Bead Array analysis showed that the MZ B cells released a large amount of IL-6, together with low amounts of TNFα and IL-10 after PGN stimulation (**Figure 2D**). Next, to test the contribution of MZ B cells to the elevated IL-6 production following systemic *S. aureus* infection, we collected tissue fluid from WT and RBP-J CKO mouse spleens at various timepoints. Notably, the levels of IL-6 in WT mouse spleens were significantly higher than those in the spleens of RBP-J CKO mice at 3 and 6 h post-infection (**Figure 2E**). Consistent with this finding, we found a significantly elevated release of IL-6 by MZ B cells at 3 h after treatment with heat-killed *S. aureus* in culture (**Supplementary Figure 2A**). Moreover, intracellular IL-6 staining by flow cytometric analysis demonstrated that the frequency of IL-6-producing MZ B cells increased significantly after systemic *S. aureus* infection *in vivo* (**Supplementary Figures 2B, C**). Together, these results suggest that MZ B cells are a source of IL-6 in response to systemic *S. aureus* infection.

Neutrophils Express Increased Levels of Multiple Chemokine Receptors After *S. aureus* Infection

Because IL-6 regulates systemic inflammatory responses partially by assisting with the infiltration of splenic leukocytes (46, 47), we next examined whether MZ B cells affect the recruitment of circulating neutrophils after systemic *S. aureus* infection. We first examined whether splenic neutrophils changed the expression of cytokine/chemokine genes after systemic *S. aureus* infection. Neutrophils isolated from untreated mice or mice infected with *S. aureus* 4 h previously were subjected to a cDNA microarray analysis. The results reveal that 24 chemokine genes were upregulated or downregulated by at least 1.5 folds in splenic neutrophils after infection (**Figure 3A** and **Supplementary Data Sheet 1**). CXCL1 and CXCL2 are members of the CXC chemokine family, and they bind with the CXC chemokine receptors CXCR1 and/or CXCR2 (48, 49). Our RT-qPCR results confirm that the *Cxcr1* and *Cxcr2* mRNA levels were significantly increased within 24 h after *S. aureus* infection (**Figure 3B**). Together, these data imply that the upregulated *Cxcr1*/*Cxcr2* in neutrophils may be a response to the enhanced expression of *Cxcl1*/*Cxcl2* in MZ B cells during the early recruitment phase after systemic *S. aureus* infection. In support of this possibility, we observed a significant increase in the percentage of splenic neutrophils soon after *S. aureus* infection in WT mice (**Figure 3C**). Notably, compared with *S. aureus*-infected WT mice, similarly infected RBP-J CKO mice had lower percentages of splenic neutrophils at 3, 6, and 24 h post-infection

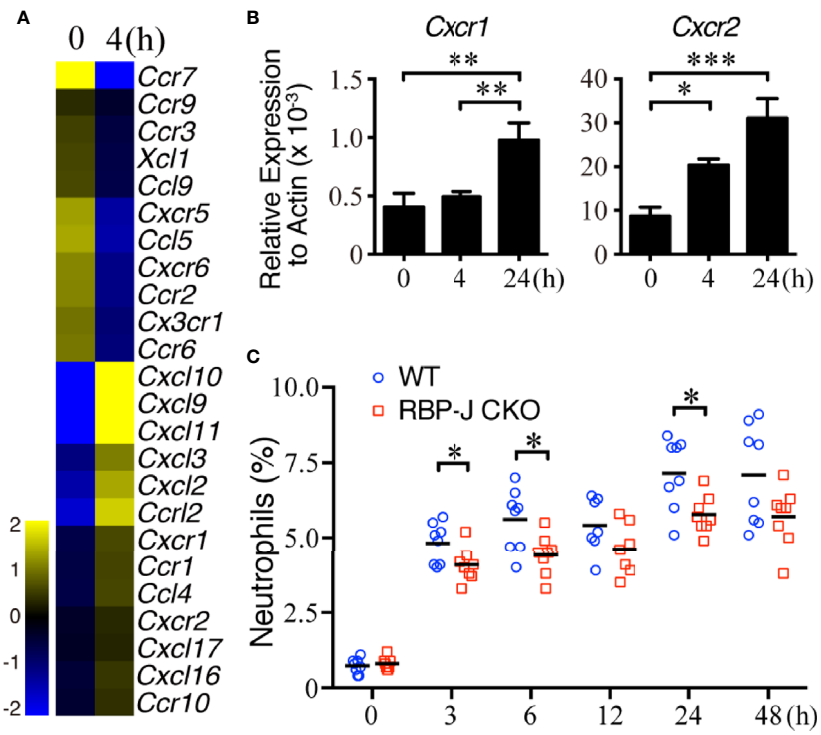

FIGURE 3 | *Cxcr1/Cxcr2* levels in splenic neutrophils after systemic *S. aureus* infection. **(A)** Heatmap from a cDNA microarray analysis showing the changes in chemokine-related genes between splenic neutrophils isolated before and 4 h after infection with *S. aureus* (2.5×10^6 CFU) by iv injection. The expression of chemokine-related genes with fold changes of >1.5 is shown. **(B)** RT-qPCR analysis showing the expression of *Cxcr1/Cxcr2* mRNA. Splenic neutrophils were isolated at 0, 4, or 24 h after *S. aureus* infection. Results were normalized to the actin mRNA levels. Three independent experiments were performed. Data are presented as the mean ± SEM (n = 6–8 mice per group). One-way ANOVA was used for comparison. **(C)** Flow cytometric analysis showing the frequency of splenic neutrophils ($Ly6G^{hi}CD11b^{hi}$) in WT and RBP-J CKO mice at various timepoints after *S. aureus* (2.5×10^6 CFU) infection. Data are presented as the mean ± SEM (n = 7–8). An unpaired two-tailed *t*-test was performed. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(**Figure 3C** and **Supplementary Figure 3**), suggesting that CXCL1/CXCL2 released by MZ B cells may recruit neutrophils in the early phase of systemic *S. aureus* infection.

IL-6 and CXCL1/CXCL2 Are Important for Neutrophil Swarming to the MZ Area in Response to Systemic *S. aureus* Infection

We next sought to examine the influence of cytokines and chemokines, on the dynamics of neutrophil infiltration into the spleen after *S. aureus* infection. We first employed confocal imaging using thick sections to define the location of the MZ B-cell area, distinguished by $CD1d^+$ in staining; it was located at the junction of the red pulp and white pulp of the spleen, separated from the follicle area (**Supplementary Figure 4A**). The number of neutrophils, as defined by $Ly6G^+$ staining, in the red pulp was small in the uninfected mice (**Figure 4A**). Notably, we observed that neutrophils swarmed and packed closely together with MZ B cells in the spleens of WT mice at 3 h post-infection. This enhanced amount of neutrophil accumulation was reduced in RBP-J CKO mice, indicating that MZ B cells are important for the early recruitment of neutrophils during systemic *S. aureus* infection (**Figure 4A**).

Because the number of neutrophils at the outer ring of the MZ declined at 12 and 24 h after systemic *S. aureus* infection

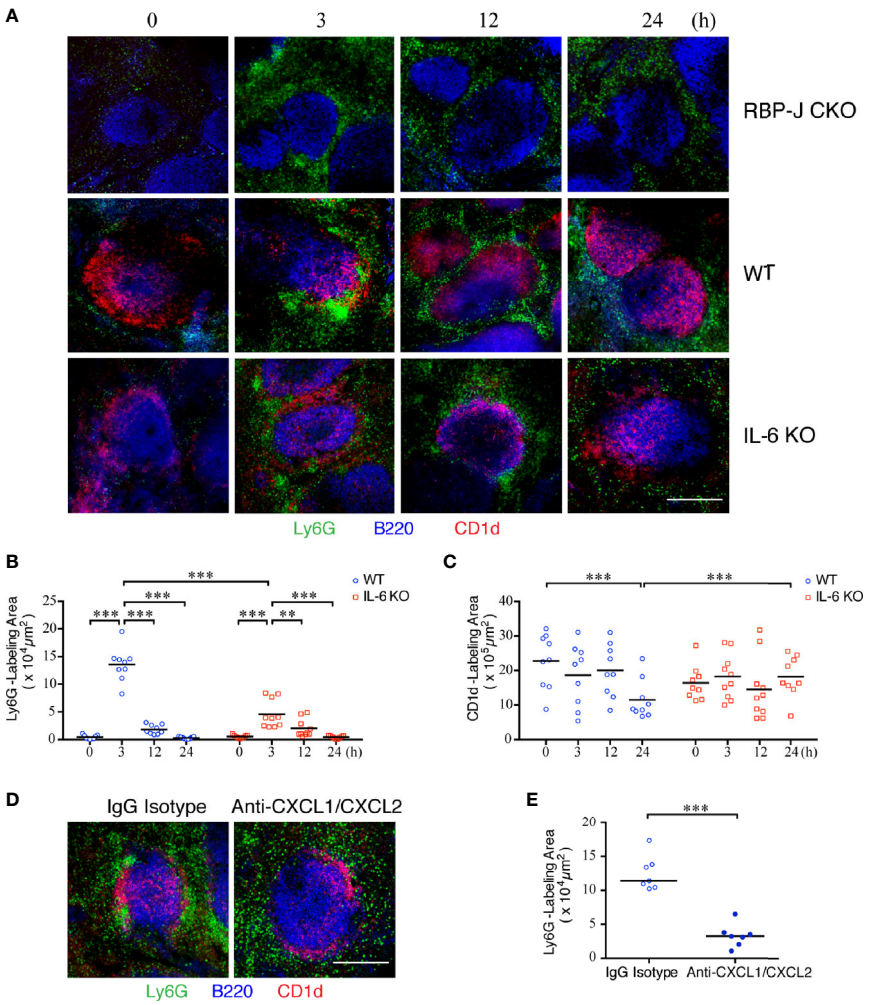

FIGURE 4 | Kinetics of neutrophil swarming and regression in the MZ zone after *S. aureus* infection. **(A)** Confocal images of immunofluorescent staining of thick spleen sections showing the distribution of MZ B cells (red) and neutrophils (green) at 0, 3, 12, or 24 h after systemic *S. aureus* (2.5×10^6 CFU) infection in RBP-J CKO, WT, or IL-6 KO mice. Scale bar = 200 μm. **(B, C)** MetaMorph software was used to calculate the area fluorescently labeled area by Ly6G per CD1d positive area **(B)** or CD1d **(C)** inside the white pulp of WT or IL-6 KO mice from the confocal microscopy images in **(A)**. **(D)** Confocal images of immunofluorescence staining of spleen sections at 3 h after systemic *S. aureus* (2.5×10^6 CFU) infection showing the numbers of neutrophils (green) in WT mice intravenously injected with anti-CXCL1/CXCL2 or IgG control antibodies one h prior to infection. Scale bar = 200 μm. **(E)** MetaMorph software was used to calculate the Ly6G fluorescently labeled area of each CD1d-positive area in the white pulp of WT mice from the confocal microscopy images in **(D)**. Results were analyzed by performing an unpaired *t*-test. Data are presented as the mean ± SEM (n = 9–10). $^{**}p < 0.01$, and $^{***}p < 0.001$.

(**Figure 4A**), when we observed that the IL-6 levels also reduced after *S. aureus* infection (**Figure 2E**), we speculated that the formation of a large number of neutrophil clusters may occur in response to the elevated expression of IL-6 in MZ B cells. In support of this idea, the number of neutrophils in *Il-6* knockout (IL-6 KO) mice at 3 h post-infection appears to be lower than that in WT mice (**Figure 4A**). A quantitative analysis performed by using MetaMorph software further revealed that the total area of neutrophil accumulation near the MZ area was significantly higher at 3 h post-infection in WT mice as compared with those at later timepoints (**Figure 4B** and **Supplementary Figure 4B**). Notably, the total area of neutrophil clustering near the MZ area was smaller in IL-6 KO mice compared with that in WT mice (**Figure 4B** and **Supplementary Figure 4C**), indicating that IL-6 promotes the initial neutrophil swarming to MZ areas during systemic *S. aureus* infection. Furthermore, the CD1d-labeled area in the spleens of WT mice was significantly smaller at 24 h post-infection as compared with that in uninfected mice (**Figure 4C**). However, in IL-6 KO mice, the size of the CD1d-labeled area was not significantly different between uninfected and *S. aureus*-infected mice, suggesting that IL-6 may affect the activation and differentiation of MZ B cells. To test whether chemotactic signals also contribute to neutrophil recruitment during systemic *S. aureus* infection, we injected anti-CXCL1 and anti-CXCL2 antibodies to neutralize the effect of CXCL1/CXCL2 one h before systemic *S. aureus* infection. Confocal imaging with thick tissue sections showed that, following depletion of the activity of CXCL1/CXCL2, neutrophils were distributed in the red pulp and much less neutrophils were present in the MZ area 3 h after *S. aureus* infection, as compared with those in infected mice injected with isotype control antibody (**Figure 4D**). A quantitative analysis performed by using MetaMorph software indicated that depletion of CXCL1/CXCL2 before infection prevents neutrophil recruitment to the MZ (**Figure 4E** and **Supplementary Figure 4D**). Together, these results indicate that IL-6 and CXCL1/CXCL2 contribute to neutrophil swarming during the early phase of systemic *S. aureus* infection.

The Neutrophil–MZ B Cell Interaction Promotes IgM Production

Because our results from the neutrophil staining in thick spleen tissue sections demonstrated a suppressed neutrophil accumulation in the MZ area of RBP-J CKO mice, we next examined the molecular consequence of an interaction between neutrophils and MZ B cells. The results from CD1d and Ly6G staining show that neutrophil clusters were in close contact with MZ B cells at 3 h after systemic *S. aureus* infection, whereas neutrophils were scattered around the outer ring of MZ B cells at 24 h post-infection (**Figure 5A**). Furthermore, confocal images of the locations of neutrophils and MZ B cells in a thick tissue section of WT spleen from a mouse infected with *S. aureus* 3 *h* previously showed a colocalization rate of 47% (**Supplementary Figures 5A** and **5B**). To further understand the consequence of the interaction between neutrophils and MZ B cells, we conducted co-culture experiments and examined whether an interaction between neutrophils and MZ B cells promotes antibacterial responses (**Figure 5B**). First, we performed a flow cytometry analysis to measure the expression levels of the costimulatory molecule CD86 and the early activation molecule CD69 on MZ B cells after they had been co-cultured with neutrophils for 3 h in the presence or absence of PGN stimulation (**Figure 5C**). We found that the CD86 and CD69 expressions on MZ B cells were elevated after PGN stimulation and that CD69 elevation was further promoted in the presence of neutrophils (**Figure 5D**). However, treatment with heat-killed *S. aureus* significantly elevated the expression of CD69 and CD86 on MZ B cells, even in the absence of neutrophils (**Supplementary Figures 6A–C**). We next assessed the interaction between neutrophils and MZ B cells by using a FLIM-FRET analysis. Confocal imaging indicated that an enrichment of CD19-labeled signal clustered on the surface of MZ B cells at the neutrophil junction following PGN stimulation (**Figure 5E**, red circle). We used the average photon arrival time to represent the mean lifetime. Our results reveal that neutrophils (donor) in contact with an MZ B cell (acceptor) indeed have a shorter fluorescent lifetime compared with neutrophils not in contact with an MZ B cell. A quantitative analysis of the FRET efficiency estimates it at 73.9% (**Figure 5E**). These results suggest that there is a direct interaction between neutrophils and MZ B cells. The amount of IgM produced by activated MZ B cells after their co-culture with neutrophils was also measured by ELISA. We found that MZ B cells co-cultured with neutrophils rapidly (within a day) released significantly higher amounts of IgM after stimulation with PGN compared with similarly treated MZ B cells cultured alone (**Figure 5F**). Similarly, co-culture of neutrophils with MZ B cells significantly enhances the production of IgM after the treatment with heat-killed *S. aureus* (**Supplementary Figure 6D**). Together, these combined results provide evidence for the direct interaction of neutrophils and MZ B cells and for the enhanced differentiation of IgM-producing cells by neutrophils after *S. aureus* infection.

Neutrophil Depletion Affects MZ B-Cell Differentiation After Bacterial Infection

To confirm whether neutrophils have important roles in assisting with MZ B-cell activity during the early phase of systemic *S. aureus* infection, we next depleted neutrophils and assessed whether the MZ B-cell differentiation was affected after *S. aureus* infection (**Figure 6A**). Mice injected with anti-Ly6G antibody showed a robust reduction of neutrophils as compared with mice injected with the isotype control antibody (**Figure 6B**). ELISAs were performed on splenic tissue fluids to analyze the amount of *S. aureus*-specific IgM at 4 and 24 h post-infection. Our results show a slightly induced production of *S. aureus*-specific IgM in control mice at 24 h post-infection, whereas mice depleted of neutrophils had significantly lower levels of *S. aureus*-specific IgM at 24 h post-infection (**Figure 6C**). Thus, neutrophils play an important role in promoting the activation and differentiation of MZ B cells during acute *S. aureus* infection.

DISCUSSION

Previous studies have noted that MZ B cells not only participate in innate immune responses but also mediate T cell-dependent immunity (50). Our findings here demonstrate the role of MZ B

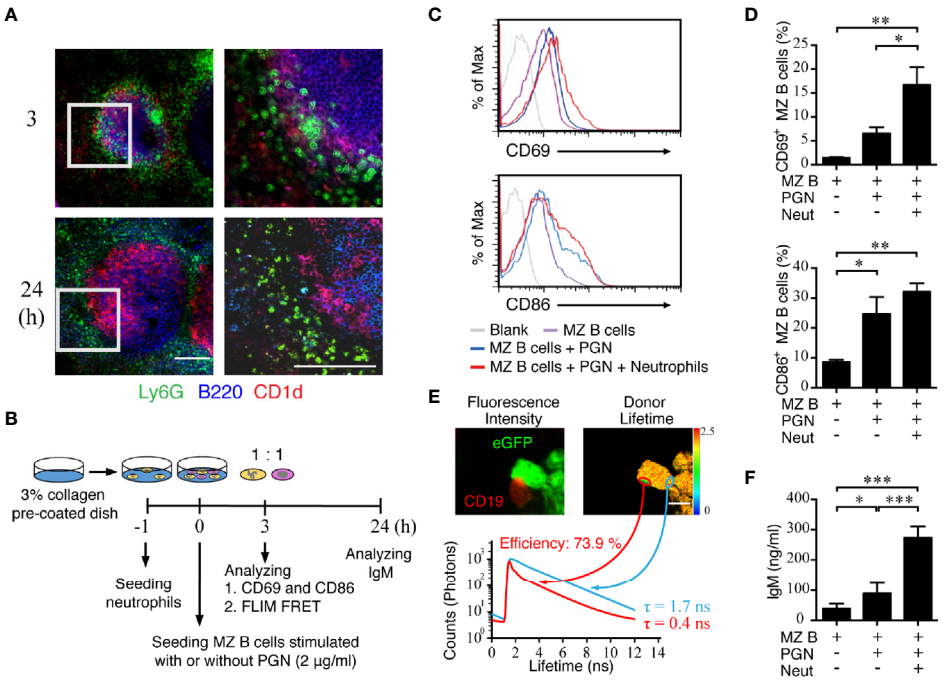

FIGURE 5 | Activation of MZ B cells and IgM production after interaction with neutrophils in *S. aureus* infection. **(A)** Confocal microscopy images showing the neutrophils and MZ B cells at 3 h or 24 h after systemic infection with *S. aureus* (2.5×10^6 CFU) in WT mice. Right panels present enlarged images from the boxed areas in the left panels. Scale bar = 100 μm. **(B)** Schematic diagram showing the experimental design of MZ B cell and neutrophil co-culture experiments. **(C)** Flow cytometric analysis showing the levels of CD69 (upper panel) and CD86 (lower panel) expression on MZ B cells co-cultured with neutrophils at 3 h after stimulation with PGN (2 μg/ml). **(D)** Statistical analysis of the percentages of $CD69^+$ (upper panel) or $CD86^+$ (lower panel) MZ B cells co-cultured with neutrophils (Neut) with or without PGN stimulation. **(E)** The interaction of neutrophils and MZ B cells detected by FLIM FRET analysis. Neutrophils were purified from LysM-eGFP mice. Sorted MZ B cells were labeled with anti-CD19 primary antibody and Alexa Fluor 546 secondary antibody. The confocal fluorescence intensity (left panel) and average donor lifetime (right panel) of neutrophils were measured. Lifetime values were pseudocolored according to the color scale. Histograms show the lifetime values of the junction area of neutrophils and MZ B cells (red histogram) as compared with neutrophils only (blue histogram), in a representative experiment. Scale bar = 5 μm. **(F)** ELISA showing the levels of IgM in supernatants of MZ B cells co-cultured with neutrophils for 24 h with or without PGN stimulation. Results were analyzed by an unpaired *t*-test. Data are analyzed by one-way ANOVA and presented as the mean ± SEM (n = 4 in D, and n = 6 in F). $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

cells in the regulation of innate immunity during systemic bacterial infection, which involves in their interaction with neutrophils. We show here that, compared with WT mice, MZ B cell-deficient mice exhibited exacerbated systemic bacterial infections with reduced survival rates, which was linked with their reduced neutrophil accumulation during the early phase of systemic *S. aureus* infection.

We here use RBP-J CKO mice to study the role of MZ B cells in systemic *S. aureus* infection. Although activation of Notch pathway has been shown to promote B cell differentiation (51), RBP-J CKO mice did not show obvious changes in Ig production in response to model antigen immunization, as compared with those produced in control mice (3). Therefore, the reduced production of IgM after systemic *S. aureus* infection in RBP-J CKO mice may not result from the effects of altering Notch signaling during plasma cell differentiation. Also, the reduced IgM production after systemic *S. aureus* infection in RBP-J CKO mice may not attribute to the changes in the population of macrophages because RBP-J CKO mice still showed the normal presence of metallophilic macrophages adjacent to the MZ in the spleen (3, 52). We found that IL-6 is important for neutrophil swarming during systemic *S. aureus* infection and that MZ B cells may be an important cellular source of IL-6 in this context. The roles of IL-6 in B-cell immunity have been extensively studied. For example, in combination with a proliferation-inducing ligand (APRIL) or stem cell-soluble factor, IL-6 can promote the production of immunoglobulin by long-lived plasma cells in the bone marrow (53, 54). IL-6 produced by B cells also contributes to the formation of spontaneous germinal centers in autoimmunity (55). Here, we observed that IL-6 levels in the spleen were largely reduced in RBP-J CKO mice, compared with WT mice, during the early phase of systemic *S. aureus* infection. We suspect that this robust reduction in IL-6 production might at least partly result from the lack of MZ B cells. However, it has been shown that IL-6-driven signaling restrains the recruitment of neutrophils in an animal model of acute peritoneal inflammation. STAT3 activation mediated by gp130 reduces the expression of neutrophil-activating chemokines CXCL1/KC and affects neutrophil clearance (56). IL-6 produced by MZ B cells accounts for the proinflammatory

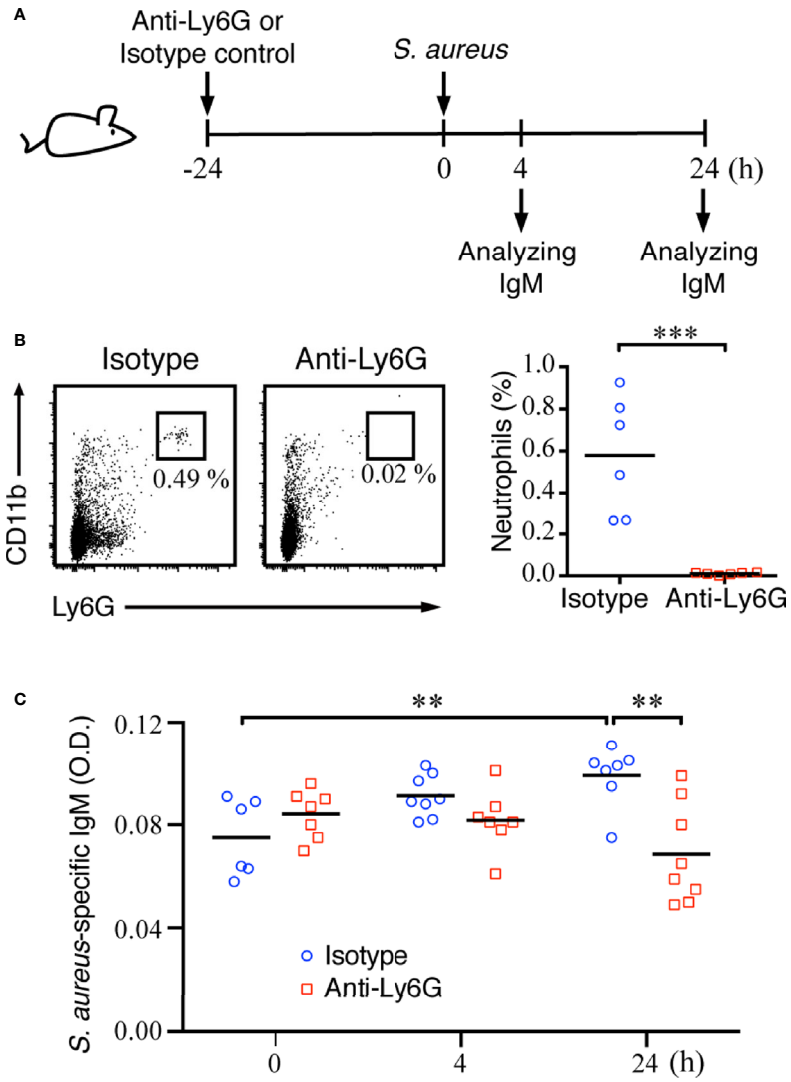

FIGURE 6 | Effect of neutrophil deficiency on the activation of MZ B cells and IgM production in *S. aureus*-infected mice. **(A)** Schematic diagram of the experimental design. **(B)** Flow cytometry analysis showing the depletion of neutrophils ($Ly6G^{hi}CD11b^{hi}$) in the spleens of C57BL/6 mice at 24 h after an intraperitoneal injection of anti-Ly6G antibody. **(C)** ELISA analysis showing the levels of *S. aureus*-specific IgM in the splenic tissue fluids of C57BL/6 mice injected with isotype control antibody or anti-Ly6G antibody. Results were analyzed by an unpaired Student's *t*-test. Data are presented as the mean ± SEM (n = 6 in B, and n = 6–8 in C). $^{**}p < 0.01$ and $^{***}p < 0.001$.

role of MZ B cells during endotoxic shock through IgM Fc receptor (FcμR)-coupled TLR4 signaling (13). In the present study, PGN from *S. aureus* may initiate TLR2-mediated signaling to induce IL-6 production by MZ B cells. We show that IL-6 is beneficial for the hosts during systemic *S. aureus* infection because IL-6 has a positive role in neutrophil swarming during the acute phase. It is still poorly understood how IL-6 signaling can act as a double-edged sword to regulate the innate immune responses under different conditions. Here, we speculate that following *S. aureus* infection, activated MZ B cells may release the chemokine CXCL2 to attract many neutrophils that have upregulated expression of their CXCR2 receptor, *Cxcr1*/*Cxcr2*. In support of this possibility, we found a correlation between lower numbers of neutrophils surrounding the MZ area with lower levels of CXCL2 expression by MZ B cells at 24 h after systemic *S. aureus* infection. We further demonstrated that CXCL1/CXCL2 released by MZ B cells also participated in the recruitment of neutrophils after systemic *S. aureus* infection. Prior studies have noted that CXCL1/CXCL2 are the main neutrophil-attracting chemokines in group B Streptococcus (GBS)-induced neutrophil recruitment. Specifically, neutrophils can employ a positive feedback mechanism driven by high levels of CXCL2 to enhance their recruitment to the sites of infection and antibacterial activity (57). We suspect that the increased

CXCL1/CXCL2 expression by neutrophils after *S. aureus* infection may also act in an autocrine manner to attract more neutrophils and enhance antibacterial activities.

Neutrophils are a heterogeneous and plastic innate cell population. Inflammatory cytokines, including GM-CSF, or co-culture with antigen and T cells can induce the expression of MHCII on neutrophils, thereby triggering the antigen-presenting functions of neutrophils (58, 59). However, the underlying molecular mechanisms for antigen presentation crosstalk between neutrophils and MZ B cells remain unclear. Previous studies using FRET analysis reported that antibody-induced signals from Transmembrane Activator and CAML Interactor (TACI) and TLR synergistically activate MZ B cells and induce plasmablast differentiation through a rapamycin-sensitive pathway (60). For a long time, it has been reported that MZ B cells integrate signals from B-cell receptors (BCR), complement receptors (C3), and Toll-like receptors to rapidly activate antigen-specific IgM (8, 61). In the co-culture experiment, compared with PGN stimulated MZ B cells, we found that treatment with heat-killed *S. aureus* was able to effectively increase the expression of CD69 and CD86 on MZ B cells. We speculate that, in addition to activating TLR2 on MZ B cells through PGN, *S. aureus* contains other molecules to be recognized by MZ B cells. However, differentiation of MZ B cells into Ig producing cells needs the help from neutrophils. Here, we performed a FLIM-FRET analysis by using confocal microscopy, the results of which demonstrate an interaction between MZ B cells and neutrophils at 3 h after stimulation with the cell wall component of *S. aureus*. We found that the direct cell-cell communication between activated neutrophils and MZ B cells was related to CD19 expression. However, the exact molecules involved in this direct interaction between MZ B cells and neutrophils requires further study. Nevertheless, our data, along with a previous report showing that IL-6-induced STAT3 activation enhances the recruitment of neutrophils and contributes to host defense against *E. coli*-induced pneumonia, support the idea that neutrophils possess multiple functions to assist in the clearance of bacterial infection in the spleen (62). We found that mice depleted of neutrophils were more susceptible to systemic *S. aureus* infection as compared with mice treated with isotype control antibodies. The spleens of the neutrophil-depleted mice were nearly devoid of infiltrating neutrophils, and 27.5% of these mice died within 24 h of infection with *S. aureus* (**Supplementary Figure 7**). Thus, our findings are similar to the results from a previous study demonstrating that systemic TLR2 activation and bone marrow granulocyte depletion in mice exacerbated *Listeria monocytogenes* infection and led to uncontrolled bacterial propagation (63).

The production of high affinity antibodies following infection-induced germinal center reactions usually requires several days to emerge. MZ B cells fill the gap before the peak germinal center reactions by deploying innate immune defenses and rapidly producing antibodies. The present work demonstrates the requirement for IL-6 and CXCL1/CXCL2 to attract an accumulation of neutrophils. This direct interaction between MZ B cells and neutrophils in turn assists with the maturation of MZ B cells as they differentiate into antibody-secreting cells during the early phase of bacterial infection. Thus, MZ B cells possess the regulatory functions necessary to orchestrate neutrophil swarming during the early stage of systemic bacterial infection. Our results may provide some clues on how better to control systemic *S. aureus* infection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Academia Sinica.

AUTHOR CONTRIBUTIONS

K-IL conceived and designed the study. L-WL, C-WC, M-FC, and I-YL performed the experiments and analyzed the data. L-WL and K-IL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.636818/full#supplementary-material

REFERENCES

1. Vazquez MI, Catalan-Dibene J, Zlotnik A. B Cells Responses and Cytokine Production are Regulated by Their Immune Microenvironment. *Cytokine* (2015) 74:318–26. doi: 10.1016/j.cyto.2015.02.007
2. Khan WN. B Cell Receptor and BAFF Receptor Signaling Regulation of B Cell Homeostasis. *J Immunol* (2009) 183:3561. doi: 10.4049/jimmunol.0800933
3. Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, et al. Notch-RBP-J Signaling is Involved in Cell Fate Determination of Marginal Zone B Cells. *Nat Immunol* (2002) 3:443–50. doi: 10.1038/ni793
4. Balazs M, Martin F, Zhou T, Kearney J. Blood Dendritic Cells Interact With Splenic Marginal Zone B Cells to Initiate T-independent Immune Responses. *Immunity* (2002) 17:341–52. doi: 10.1016/S1074-7613(02)00389-8
5. Lopes-Carvalho T, Kearney JF. Development and Selection of Marginal Zone B Cells. *Immunol Rev* (2004) 197:192–205. doi: 10.1111/j.0105-2896.2004.0112.x
6. Lopes-Carvalho T, Foote J, Kearney JF. Marginal Zone B Cells in Lymphocyte Activation and Regulation. *Curr Opin Immunol* (2005) 17:244–50. doi: 10.1016/j.coi.2005.04.009
7. Oliver AM, Martin F, Kearney JF. IgMhighCD21high Lymphocytes Enriched in the Splenic Marginal Zone Generate Effector Cells More Rapidly Than the Bulk of Follicular B Cells. *J Immunol* (1999) 162:7198–207.
8. Pone EJ, Zhang J, Mai T, White CA, Li G, Sakakura JK, et al. BCR-Signalling Synergizes With TLR-signalling for Induction of AID and Immunoglobulin Class-Switching Through the non-Canonical NF-kappaB Pathway. *Nat Commun* (2012) 3:767. doi: 10.1038/ncomms1769
9. Martin F, Oliver AM, Kearney JF. Marginal Zone and B1 B Cells Unite in the Early Response Against T-independent Blood-Borne Particulate Antigens. *Immunity* (2001) 14:617–29. doi: 10.1016/S1074-7613(01)00129-7
10. Lino AC, Mohr E, Demengeot J. Naturally Secreted Immunoglobulins Limit B1 and MZ B-Cell Numbers Through a Microbiota-Independent Mechanism. *Blood* (2013) 122:209–18. doi: 10.1182/blood-2012-08-447136
11. Cerutti A, Cols M, Puga I. Marginal Zone B Cells: Virtues of Innate-Like Antibody-Producing Lymphocytes. *Nat Rev Immunol* (2013) 13:118–32. doi: 10.1038/nri3383
12. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, et al. B Cell Depletion Therapy Ameliorates Autoimmune Disease Through Ablation of IL-6-Producing B Cells. *J Exp Med* (2012) 209:1001–10. doi: 10.1084/jem.20111675
13. Honda S, Sato K, Totsuka N, Fujiyama S, Fujimoto M, Miyake K, et al. Marginal Zone B Cells Exacerbate Endotoxic Shock Via Interleukin-6 Secretion Induced by Fcalpha/muR-coupled TLR4 Signalling. *Nat Commun* (2016) 7:11498. doi: 10.1038/ncomms11498
14. Wolf J, Rose-John S, Garbers C. Interleukin-6 and its Receptors: A Highly Regulated and Dynamic System. *Cytokine* (2014) 70:11–20. doi: 10.1016/j.cyto.2014.05.024
15. Choy E, Rose-John S. Interleukin-6 as a Multifunctional Regulator: Inflammation, Immune Response, and Fibrosis. *J Scleroderma Relat Disord* (2017) 2:S1–5. doi: 10.5301/jsrd.5000265
16. Tanaka T, Narazaki M, Kishimoto T. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harbor Perspect Biol* (2014) 6:a016295–a016295. doi: 10.1101/cshperspect.a016295
17. Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: A Regulator of the Transition From Neutrophil to Monocyte Recruitment During Inflammation. *Trends Immunol* (2003) 24:25–9. doi: 10.1016/S1471-4906(02)00013-3
18. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The Pro- and Anti-Inflammatory Properties of the Cytokine Interleukin-6. *Biochim Biophys Acta* (2011) 1813:878–88. doi: 10.1016/j.bbamcr.2011.01.034
19. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil Kinetics in Health and Disease. *Trends Immunol* (2010) 31:318–24. doi: 10.1016/j.it.2010.05.006
20. Iba T, Hashiguchi N, Nagaoka I, Tabe Y, Murai M. Neutrophil Cell Death in Response to Infection and its Relation to Coagulation. *J Intensive Care* (2013) 1:13–3. doi: 10.1186/2052-0492-1-13
21. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the Site of Inflammation: The Leukocyte Adhesion Cascade Updated. *Nat Rev Immunol* (2007) 7:678–89. doi: 10.1038/nri2156
22. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of Granulocyte Survival and Programmed Cell Death by Cytokines and Bacterial Products. *Blood* (1992) 80:2012–20. doi: 10.1182/blood.V80.8.2012.2012
23. Medeiros AI, Bonato VL, Malheiro A, Dias AR, Silva CL, Faccioli LH. Histoplasma Capsulatum Inhibits Apoptosis and Mac-1 Expression in Leucocytes. *Scand J Immunol* (2002) 56:392–8. doi: 10.1046/j.1365-3083.2002.01142.x
24. Rosales C. Neutrophil: A Cell With Many Roles in Inflammation or Several Cell Types? *Front Physiol* (2018) 9:113. doi: 10.3389/fphys.2018.00113
25. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity. *Nat Rev Immunol* (2011) 11:519–31. doi: 10.1038/nri3024
26. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B Cell-Helper Neutrophils Stimulate the Diversification and Production of Immunoglobulin in the Marginal Zone of the Spleen. *Nat Immunol* (2011) 13:170–80. doi: 10.1038/ni.2194
27. Parsa R, Lund H, Georgoudaki A-M, Zhang X-M, Ortlieb Guerreiro-Cacais A, Grommisch D, et al. BAFF-secreting Neutrophils Drive Plasma Cell Responses During Emergency Granulopoiesis. *J Exp Med* (2016) 213:1537–53. doi: 10.1084/jem.20150577
28. Kienle K, Lämmermann T. Neutrophil Swarming: An Essential Process of the Neutrophil Tissue Response. *Immunol Rev* (2016) 273:76–93. doi: 10.1111/imr.12458
29. Scapini P, Cassatella MA. Social Networking of Human Neutrophils Within the Immune System. *Blood* (2014) 124:710–9. doi: 10.1182/blood-2014-03-453217
30. Futosi K, Fodor S, Mócsai A. Neutrophil Cell Surface Receptors and Their Intracellular Signal Transduction Pathways. *Int Immunopharmacology* (2013) 17:638–50. doi: 10.1016/j.intimp.2013.06.034
31. Mortaz E, Alipoor SD, Adcock IM, Mumby S, Koenderman L. Update on Neutrophil Function in Severe Inflammation. *Front Immunol* (2018) 9:2171. doi: 10.3389/fimmu.2018.02171
32. Rosales C, Demaurex N, Lowell CA, Uribe-Querol E. Neutrophils: Their Role in Innate and Adaptive Immunity. *J Immunol Res* (2016) 2016:1469780–1469780. doi: 10.1155/2016/1469780
33. David MZ, Daum RS. Community-associated Methicillin-Resistant Staphylococcus Aureus: Epidemiology and Clinical Consequences of an Emerging Epidemic. *Clin Microbiol Rev* (2010) 23:616–87. doi: 10.1128/cmr.00081-09
34. Faust N, Varas F, Kelly LM, Heck S, Graf T. Insertion of Enhanced Green Fluorescent Protein Into the Lysozyme Gene Creates Mice With Green Fluorescent Granulocytes and Macrophages. *Blood* (2000) 96:719–26. doi: 10.1182/blood.V96.2.719.014k29_719_726
35. Cossarizza A, Chang HD, Radbruch A, Akdis M, Andrä I, Annunziato F, et al. Guidelines for the Use of Flow Cytometry and Cell Sorting in Immunological Studies. *Eur J Immunol* (2017) 47:1584–797. doi: 10.1002/eji.201646632
36. Swamydas M, Luo Y, Dorf ME, Lionakis MS. Isolation of Mouse Neutrophils. *Curr Protoc Immunol* (2015) 110:3.20.1–15. doi: 10.1002/0471142735.im0320s110
37. Calzetti F, Tamassia N, Arruda-Silva F, Gasperini S, Cassatella MA. The Importance of Being "Pure" Neutrophils. *J Allergy Clin Immunol* (2017) 139:352–355.e6. doi: 10.1016/j.jaci.2016.06.025
38. Snippert HJ, Schepers AG, Delconte G, Siersema PD, Clevers H. Slide Preparation for Single-Cell–Resolution Imaging of Fluorescent Proteins in Their Three-Dimensional Near-Native Environment. *Nat Protoc* (2011) 6:1221–8. doi: 10.1038/nprot.2011.365
39. Shim K. Vibratome Sectioning for Enhanced Preservation of the Cytoarchitecture of the Mammalian Organ of Corti. *J Vis Exp JoVE* (2011) 52:2793. doi: 10.3791/2793
40. Clancy B, Cauller LJ. Reduction of Background Autofluorescence in Brain Sections Following Immersion in Sodium Borohydride. *J Neurosci Methods* (1998) 83:97–102. doi: 10.1016/s0165-0270(98)00066-1
41. Racine R, Winslow GM. IgM in Microbial Infections: Taken for Granted? *Immunol Lett* (2009) 125:79–85. doi: 10.1016/j.imlet.2009.06.003
42. Tellier J, Nutt SL. Standing Out From the Crowd: How to Identify Plasma Cells. *Eur J Immunol* (2017) 47:1276–9. doi: 10.1002/eji.201747168
43. Lund FE. Cytokine-Producing B Lymphocytes-Key Regulators of Immunity. *Curr Opin Immunol* (2008) 20:332–8. doi: 10.1016/j.coi.2008.03.003
44. Zhiming W, Luman W, Tingting Q, Yiwei C. Chemokines and Receptors in Intestinal B Lymphocytes. *J Leukoc Biol* (2018) 103:807–19. doi: 10.1002/jlb.1ru0717-299rr

45. Vollmer W, Blanot D, De Pedro MA. Peptidoglycan Structure and Architecture. *FEMS Microbiol Rev* (2008) 32:149–67. doi: 10.1111/j.1574-6976.2007.00094.x
46. Vardam TD, Zhou L, Appenheimer MM, Chen Q, Wang W-C, Baumann H, et al. Regulation of a lymphocyte-endothelial-IL-6 Trans-Signaling Axis by Fever-Range Thermal Stress: Hot Spot of Immune Surveillance. *Cytokine* (2007) 39:84–96. doi: 10.1016/j.cyto.2007.07.184
47. Li Y, Wu J, Xu L, Wu Q, Wan Z, Li L, et al. Regulation of Leukocyte Recruitment to the Spleen and Peritoneal Cavity During Pristane-Induced Inflammation. *J Immunol Res* (2017) 2017:9891348–9891348. doi: 10.1155/2017/9891348
48. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, et al. CXC Chemokines in Angiogenesis. *J Leukoc Biol* (2000) 68:1–8.
49. Graves DT, Jiang Y. Chemokines, a Family of Chemotactic Cytokines. *Crit Rev Oral Biol Med* (1995) 6:109–18. doi: 10.1177/10454411950060020101
50. Zouali M, Richard Y. Marginal Zone B-cells, a Gatekeeper of Innate Immunity. *Front Immunol* (2011) 2:63. doi: 10.3389/fimmu.2011.00063
51. Santos MA, Sarmento LM, Rebelo M, Doce AA, Maillard I, Dumortier A, et al. Notch1 Engagement by Delta-like-1 Promotes Differentiation of B Lymphocytes to Antibody-Secreting Cells. *Proc Natl Acad Sci* (2007) 104:15454–9. doi: 10.1073/pnas.0702891104
52. Zhang Z, Zhou L, Yang X, Wang Y, Zhang P, Hou L, et al. Notch-RBP-J-independent Marginal Zone B Cell Development in IgH Transgenic Mice With VH Derived From a Natural Polyreactive Antibody. *PloS One* (2012) 7: e38894. doi: 10.1371/journal.pone.0038894
53. Hirano T, Taga T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K, et al. Purification to Homogeneity and Characterization of Human B-cell Differentiation Factor (BCDF or Bsfp-2). *Proc Natl Acad Sci U S A* (1985) 82:5490–4. doi: 10.1073/pnas.82.16.5490
54. Jourdan M, Cren M, Robert N, Bolloré K, Fest T, Duperray C, et al. IL-6 Supports the Generation of Human Long-Lived Plasma Cells in Combination With Either APRIL or Stromal Cell-Soluble Factors. *Leukemia* (2014) 28:1647–56. doi: 10.1038/leu.2014.61
55. Arkatkar T, Du SW, Jacobs HM, Dam EM, Hou B, Buckner JH, et al. B Cell-Derived IL-6 Initiates Spontaneous Germinal Center Formation During Systemic Autoimmunity. *J Exp Med* (2017) 214:3207–17. doi: 10.1084/jem.20170580
56. Fielding CA, McLoughlin RM, McLeod L, Colmont CS, Najdovska M, Grail D, et al. IL-6 Regulates Neutrophil Trafficking During Acute Inflammation Via STAT3. *J Immunol* (2008) 181:2189–95. doi: 10.4049/jimmunol.181.3.2189
57. Lentini G, Famà A, Biondo C, Mohammadi N, Galbo R, Mancuso G, et al. Neutrophils Enhance Their Own Influx to Sites of Bacterial Infection Via Endosomal Tlr-Dependent Cxcl2 Production. *J Immunol* (2020) 204:660. doi: 10.4049/jimmunol.1901039
58. Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC Class II on Human Polymorphonuclear Neutrophils by Granulocyte/Macrophage Colony-Stimulating Factor, IFN-gamma, and IL-3. *J Immunol* (1993) 151:1482–90.
59. Radsak M, Iking-Konert C, Stegmaier S, Andrassy K, Hänsch GM. Polymorphonuclear Neutrophils as Accessory Cells for T-cell Activation: Major Histocompatibility Complex Class II Restricted Antigen-Dependent Induction of T-cell Proliferation. *Immunology* (2000) 101:521–30. doi: 10.1046/j.1365-2567.2000.00140.x
60. Sintes J, Gentile M, Zhang S, Garcia-Carmona Y, Magri G, Cassis L, et al. mTOR Intersects Antibody-Inducing Signals From TACI in Marginal Zone B Cells. *Nat Commun* (2017) 8:1462. doi: 10.1038/s41467-017-01602-4
61. Guinamard R, Okigaki M, Schlessinger J, Ravetch JV. Absence of Marginal Zone B Cells in Pyk-2–deficient Mice Defines Their Role in the Humoral Response. *Nat Immunol* (2000) 1:31–6. doi: 10.1038/76882
62. Jones MR, Quinton LJ, Simms BT, Lupa MM, Kogan MS, Mizgerd JP. Roles of Interleukin-6 in Activation of STAT Proteins and Recruitment of Neutrophils During Escherichia Coli Pneumonia. *J Infect Dis* (2006) 193:360–9. doi: 10.1086/499312
63. Navarini AA, Lang KS, Verschoor A, Recher M, Zinkernagel AS, Nizet V, et al. Innate Immune-Induced Depletion of Bone Marrow Neutrophils Aggravates Systemic Bacterial Infections. *Proc Natl Acad Sci U S A* (2009) 106:7107–12. doi: 10.1073/pnas.0901162106

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IL-4-Responsive B Cells Are Detrimental During Chronic Tuberculosis Infection in Mice

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In tuberculosis, T cell-mediated immunity is extensively studied whilst B cells received limited attention in human and mice. Of interest, *Mycobacterium tuberculosis* (*Mtb*) does increase IL-4 Receptor-alpha (IL4Rα) expression in murine B cells. To better understand the role of IL4Rα signalling in B cells, we compared wild type mice with B cell-specific IL4Rα deficient mice (mb1^{cre}IL-4Rα$^{-/lox}$ mice). Chronic *Mtb* aerosol infection in mb1^{cre}IL-4Rα$^{-/lox}$ mice reduced lung and spleen bacterial burdens, compared to littermate (IL-4Rα$^{-/lox}$) control animals. Consequently, lung pathology, inflammation and inducible nitric oxide synthase (iNOS) expression were reduced in the lungs of mb1^{cre}IL-4Rα$^{-/lox}$ mice, which was also accompanied by increased lung IgA and decreased IgG1 levels. Furthermore, intratracheal adoptive transfer of wild-type B cells into B cell-specific IL4Rα deficient mice reversed the protective phenotype. Moreover, constitutively mCherry expressing *Mtb* showed decreased association with B cells from mb1^{cre}IL-4Rα$^{-/lox}$ mice *ex vivo*. In addition, supernatants from *Mtb*-exposed B cells of mb1^{cre}IL-4Rα$^{-/lox}$ mice also increased the ability of macrophages to produce nitric oxide, IL-1β, IL-6 and TNF. Together, this demonstrates that IL-4-responsive B cells are detrimental during the chronic phase of tuberculosis in mice with perturbed antibody profiles, inflammatory cytokines and *tnf* and *stat1* levels in the lungs.

Keywords: B cells, TB, Mice (balb/c), human, IL-4RA

INTRODUCTION

B cells are well established as antibody-producing cells critical for the humoral arm of adaptive immunity against a variety of infections. Emerging results uncover more complex antibody-independent involvement of B cells in regulation and effector functions, influencing the outcome of the disease. B cells can process and present antigens, act as accessory cells and produce cytokines that prime other immune cells critical for immunity against infections (1, 2). Like T cells, studies showed that B cells are classified as "regulatory" and "effector" B cell subsets based on the cytokines they produce (3). Regulatory B cells produce TGF-β and IL-10, important in colitis (4), arthritis (5) and allergic airway inflammation (6, 7) and tuberculosis (8). Effector Be1 cells produce IFN-γ (9), IL-12, TNF during Th1 driving infections such as *Leishmania major* (10), *Toxoplasma gondii* (3). Effector Be2 (11) cells produce IL-2, IL-4, IL-13 during Th2 driving *Heligmosomoides polygyrus* (12) and *Nippostrongylous brasiliensis* (13) infections. Thus, B cell-driven cytokines drive host beneficial or detrimental response during type 1 and type 2 infections.

In tuberculosis, B cells are present in lymphoid clusters in mouse (14), non-human primate (15) and human tuberculous granulomas (14, 16, 17). B cells participate in orchestrating granuloma formation is revealed by studies of targeted depletion using either anti-CD20 antibody/rituximab (18) or B cell-deficient mice (19, 20). However; the variations during tuberculosis outcome ranges from B cells being redundant (21), delay immune responses (22) and control lung pathology (19). Moreover, studies showed the role of B cells in granulomatous inflammatory responses by controlling neutrophilia and Th17 responses (23), IL-10 regulation and consequent host protection (19, 24). However, in cynomolgus macaques, B cell depletion using rituximab showed no influence on the outcome of tuberculosis disease (25). Such global depletion approaches masked distinct B cell functions, the local effects of B cells, the contribution of B cell subsets and their secreted cytokines in shaping immune responses necessary for the control of tuberculosis. Hence, such broad approaches from these studies failed to identify a major role of B cells in tuberculosis. Apart from B cells, in patients with active pulmonary tuberculosis, IL-4 secretion from BAL cells revealed a strong association with acid-fast *Mycobacterium tuberculosis* bacilli staining in sputum smear (26), suggesting a permissive Th2 environment at the site of infection. In some studies, IL-4 was shown to predict the development of active TB disease in exposed healthcare workers and household contacts (27, 28). We have previously shown that the disruption of IL-4Rα signalling in macrophages/neutrophils did not play a role in TB disease progression in mice (29). The ability of *Mtb* to induce Arginase 1 independent of IL-4Rα signalling contributed to the lack of phenotype in these mice. In a recent study, recombinant IL-4 impaired containment of *Mtb* in monocyte-derived macrophages associated with the expansion T_{reg} population amongst T_{eff} cells (30). The effect of IL-4 signalling on lymphocytic cells may be more profound than myeloid cells in tuberculosis.

Therefore, we hypothesized that the ablation of IL-4Rα signalling on B cells specifically influences the immune response and the outcome of tuberculosis disease. The present study used BALB/c mice lacking IL-4Rα specifically on B cells, mb1^{cre}IL-4Rα$^{-/lox}$, while maintaining intact receptor signalling on other cells (31). We show that the B cells lacking IL-4Rα have decreased mycobacterial burdens and lung pathology during the chronic tuberculosis infection. Importantly, adoptive transfer of IL-4Rα-sufficient B cells from wild-type donor mice abolished the protective effect in mb1^{cre}IL-4Rα$^{-/lox}$ mice. We uncovered IL-4Rα deletion on B cells decreased *tnf* and *stat1* expression and also dampened lung IFN-β production. Mechanistically, we show that the absence of IL-4Rα on B cells increased macrophage inflammatory response *ex vivo*.

METHODS

Mice

Wild-type (BALB/c), littermate control (IL-4Rα$^{-/lox}$) and B cell-specific IL-4Rα deficient mice (mb-1^{cre}IL-4Rα$^{-/lox}$) on a BALB/c background (8-12 weeks) were kept under specific-pathogen-free conditions in individually ventilated cages. The genotypes of the mice were confirmed by PCR analysis of the DNA from tail biopsies. All experiments were performed in accordance with the South African National Guidelines and University of Cape Town of practice for laboratory animal procedures.

Mtb Culture and Aerosol or Intranasal Infection in Mice

Mycobacterium tuberculosis H37Rv was grown in Middlebrook 7H9 broth as described previously (29). Prior to infection, stock solutions of *Mtb* were thawed, washed once with phosphate-buffered saline and inoculum was prepared in sterile saline. Aerosol infection was performed using an inhalation exposure system (model A4224, Glas-Col). To infect mice with a low dose of 100 CFU/lung, animals were exposed for 40 min to an aerosol generated by nebulizing approximately 6 ml of a suspension containing $2.4x10^7$ live bacteria. Similarly, for intranasal infection, 25μl per nostril was administered in anaesthetized mice to achieve the indicated dose. After infection, the inoculum was also plated to determine the change in the inoculum. Infection dose was checked at one day post-infection by determining the bacterial load in the lungs of four infected mice.

Determination of Mycobacterial Load, Histopathology and Immunohistochemistry

Mycobacterial loads in lungs and spleen of *Mtb*-infected mice were determined at different time points post-infection as previously described (29). Lungs of *Mtb*-infected mice were fixed with 4% phosphate-buffered formalin, and 3 μm-thick sections were stained with either H&E or rabbit anti-mouse antibody specific for iNOS (Abcam) or rabbit anti-mouse IgA antibody (Abcam). Detection was performed using HRP-labelled anti-rabbit antibody (Dako) followed by 3, 3'-diaminobenzidine substrate (Dako). The lung images and lesion areas, iNOS and IgA positive areas were

acquired in Nikon 90i Eclipse widefield microscope and quantified using NIS elements.

Lung Immune Cell Populations

Single-cell suspensions of the lungs were prepared as previously described (29). 1x10^6 cells were then subjected to staining for B cells (CD3$^-$CD19$^+$), CD4 T cells (CD19$^-$CD3$^+$CD4$^+$), CD8 T cells (CD19$^-$CD3$^+$CD8$^+$), macrophages (CD11c$^-$CD11b$^+$MHCII$^+$), dendritic cells (CD11b$^-$CD11c$^+$MHCII$^+$) and neutrophils (SiglecF$^-$CD11c$^-$Gr-1$^+$) in presence of 1% rat serum and 10µg/ml FcyR blocking antibody for 30min on ice. Similarly, lung B cell subsets were analyzed as B-1a (CD19$^+$B220$^+$CD43$^+$CD5^{high}IgM$^+$), B-1b (CD19$^+$B220$^+$CD43$^+$CD5^{low}IgM$^+$), B-2 (CD19$^+$B220$^+$CD43$^-$IgM$^+$IgD$^+$) B-10 (CD19$^+$B220$^+$CD43$^+$CD5$^+$CD1d$^+$), Plasma (CD19$^+$CD138$^+$MHCII^{low}CD44^{high}), Plasmablast (CD19$^+$CD138$^+$MHCII$^+$CD44^{high}), IgM (CD19$^+$B220$^+$CD43$^-$IgM$^+$) and IgD (CD19$^+$B220$^+$IgD$^+$) B cells. Cells were washed then fixed in 2% paraformaldehyde overnight and acquired by FACS LSRII (BD Pharmingen) and analysed by FlowJo (TreeStar, US). Gating strategies are provided in **Supplementary Figures 2** and **3**. Flow cytometry antibody details are provided in **Supplementary Table 1**.

Analysis of Cytokines and Antibodies in the Lung Homogenates

Lung homogenates were analysed for the IFN-β (BioLegend), IL-6 (BD Biosciences), IL-12p40 (BD BioSciences) and IL-10 (BD BioSciences) by ELISA according to manufacturers' instructions. Total IgA, IgE and IgG1 (Southern Biotech) levels are measured in lung homogenates by coating with unlabelled goat anti-mouse antibodies (1:500 dilution) and detection with alkaline phosphatase-conjugated rat anti-mouse antibodies (1:1000 dilution).

Adoptive Transfer of B Cells

A single-cell suspension of spleen from wild-type mice was prepared to stain total spleen cells using CD3, CD19 and B220 (BD Biosciences) surface markers. Double-positive B cells (CD3$^-$CD19$^+$B220$^+$) were sorted (purity ~98%) using BD FACSAria. 1 million B cells were then transferred intranasally in mb-1^{cre}IL-4Rα$^{-/lox}$ mice. Two days after the transfer, mice were infected with *Mtb* and sacrificed at 18 weeks after infection.

Gene Expression in Sorted B Cells From Chronic *Mtb* Infection

Single-cell suspensions of the lungs were prepared as described previously (29). Cells were stained for B cells (CD3$^-$CD19$^+$) and sorted with BD FACSJazz instrument. Cells were lysed in 0.5 ml of Qiazol (Qiagen) and total RNA was extracted by RNAeasy Micro kit (Qiagen). Total RNA was transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Real-time qPCR was performed with LightCycler® 480 SYBR Green I Master mix in LightCycler® 480 II (Roche). Quantitative expression analysis of *Ifnb, il10, il6, Tnf* and *Stat1* were normalized against the housekeeping gene *Hprt*, primer sequences are shown in **Supplementary Table 2**.

B Cell Infection by mCherry *Mtb* and Supernatant Transfer to *Mtb*-Infected Macrophages

CD19$^+$ bead (Miltenyi) sorted cells from naïve spleens of control littermate (IL-4Rα$^{-/lox}$) and B cell-specific IL-4Rα deficient mice (mb1^{cre}IL-4Rα$^{-/lox}$) were exposed to constitutively mCherry expressing *Mtb* for 24 hours at a multiplicity of infection 2. B cells were later analysed for mCherry, MHCII and CD124 expression by BD Fortessa. B cell supernatants were then filtered with 0.2 µm filters to remove any extracellular *Mtb*. The supernatants were transferred to *Mtb*-infected bone marrow-derived macrophages (MOI:0.5) and incubated for 3 days. Macrophage supernatants were then analysed for the indicated cytokines by ELISA and nitric oxide by Griess reagent assay.

IL4RA and Arginase Expression on Peripheral B Cells Isolated From TB Cohort

We enrolled newly diagnosed, untreated TB cases from the clinics in Ravensmead and Uitsig, Cape Town. The participants were treated with standard anti-TB drugs for six months by the clinic. For this study, we took blood at diagnosis and at the end of anti-TB treatment after 23 weeks. We also included healthy participants from the same community. Both the TB cases and the healthy controls were HIV negative. B cells were isolated by CD19 MACS beads from peripheral blood mononuclear cells and RNA was extracted using the RNEasy® Mini Kit (Qiagen, Germany) according to manufacturer's instructions. RNA was stored at -80°C prior to perform the cDNA synthesis (First Strand Kit (Qiagen, Germany) for quantitative PCR analysis.

Study Approval

The protocol was approved by the Animal Ethics Committee (AEC Permit Number: 015/040), Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa. Participant recruitment and follow up was approved by the Human Research Ethics Committee of Stelllenbosch University (N10/01/013). Written informed consent was obtained from all study participants.

Statistics

Data are represented as mean values ± SEM. Statistical analysis was performed using Student's *t*-test, two-tailed, Welch's correction with unequal variance and ordinary one-way ANOVA, defining differences between mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ as significant *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

RESULTS

IL-4Rα Deletion on B Cells Decreased *Mtb* Burdens and Lung Pathology During Chronic Infection in Mice

We assessed the role of IL-4Rα signalling on B cells using wild-type (BALB/c), littermate control (IL-4Rα$^{-/lox}$) and B cell-specific IL-4Rα (mb1^{cre}IL-4Rα$^{-/lox}$) deficient mice in a time-kinetic

manner following *Mtb* infection. At 4 weeks post-infection, mycobacterial lung burdens in mb1^{cre}IL-4Rα$^{-/lox}$ mice were similar when compared to littermate control animals (**Figure 1A**). However, at 18 weeks post-infection, mycobacterial burdens in both lungs and spleen were significantly reduced in mb1^{cre}IL-4Rα$^{-/lox}$ mice when compared to littermate controls (**Figure 1B**). Furthermore, we determined the lung inflammation by H&E and performed immunohistochemistry for iNOS expression. At 4- and 18-weeks post-infection, we found that pulmonary pathology, lesion area (**Figures 1C, D**) and iNOS expression (**Figures 1C, E**) were significantly decreased in mb1^{cre}IL-4Rα$^{-/lox}$ mice, indicating reduced lung tissue destruction during *Mtb* infection. These results show that B cell-specific IL-4Rα ablation decreased mycobacterial burden, lung inflammation and iNOS expression in mb1^{cre}IL-4Rα$^{-/lox}$ mice during chronic tuberculosis.

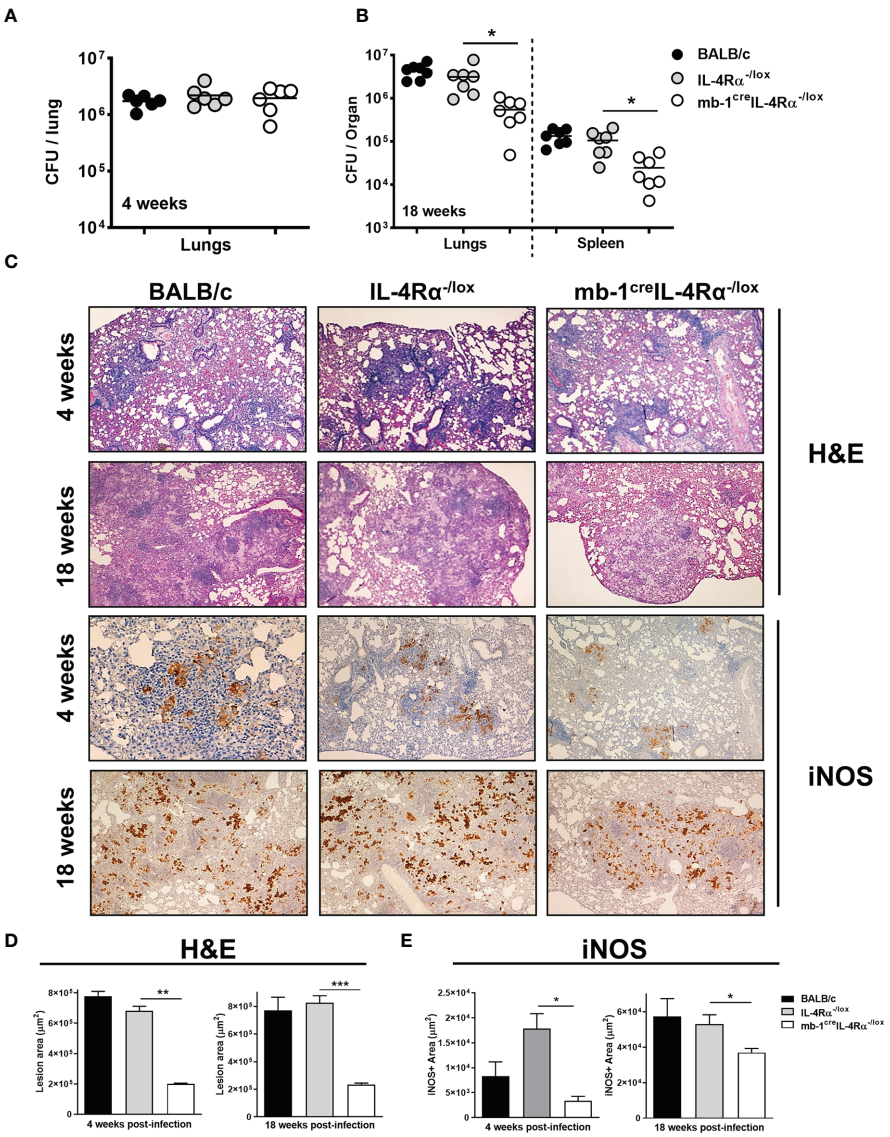

FIGURE 1 | Deletion of IL-4Rα on B cells decreased mycobacterial burdens and lung pathology during *Mtb* infection. Wild-type (BALB/c), littermate controls (IL-4Rα$^{-/lox}$) and B cell-specific IL-4Rα deficient mice (mb-1^{cre}IL-4Rα$^{-/lox}$) were infected *via* aerosol inhalation with a dose of 200 CFU H37Rv. **(A)** Mycobacterial burdens in the lungs at 4 weeks post-infection. **(B)** Lung mycobacterial burden and dissemination in the spleen at 18 weeks post-infection. **(C)** Representative histology images of lung sections stained with H&E and iNOS at 4 and 18 weeks post-infection (Original magnification: 10X). **(D, E)** Quantification of lesion area and iNOS positive area in the lungs at 4 and 18 weeks post-infection. Data are shown as mean ± SEM of n = 6 mice/group and representative of two independent experiments, analysed by unpaired, student's t-test versus littermate control, *p < 0.05, **p < 0.01 and ***p < 0.001.

Adoptive Transfer of IL-4Rα-Sufficient B Cells Abolished Decreased *Mtb* Burdens and Lung Pathology in mb1^{cre}IL-4Rα$^{-/lox}$ Mice

We investigated whether the host-protective phenotype in mb1^{cre}IL-4Rα$^{-/lox}$ mice was indeed B cell-driven. To this end, we adoptively transferred one million wild-type B cells in mb1^{cre}IL-4Rα$^{-/lox}$ mice intratracheally followed by *Mtb* infection. At 18 weeks post-infection, we found the transfer of wild-type B cells restored the lung mycobacterial burdens similar to littermate control animals (**Figure 2A**). Though the spleen mycobacterial burden was partially restored but not statistically significant, this is likely due to intratracheal B cell transfer rendered minor effect on the distal organ spleen (**Figure 2B**). We then assessed the cytokine responses in the lung homogenates, which showed that IFN-β (**Figure 2C**), IL-6 (**Figure 2D**) and IL-12p40 (**Figure 2E**) was significantly reduced whereas IL-10 (**Figure 2F**) had no effect in mb1^{cre}IL-4Rα$^{-/lox}$ mice when compared to littermate control animals. Remarkably, the adoptive transfer of wild-type B cells restored IFN-β (**Figure 2C**) production, but not IL-6 (**Figure 2D**), IL-12p40 (**Figure 2E**) and IL-10 (**Figure 2F**) in the lungs of

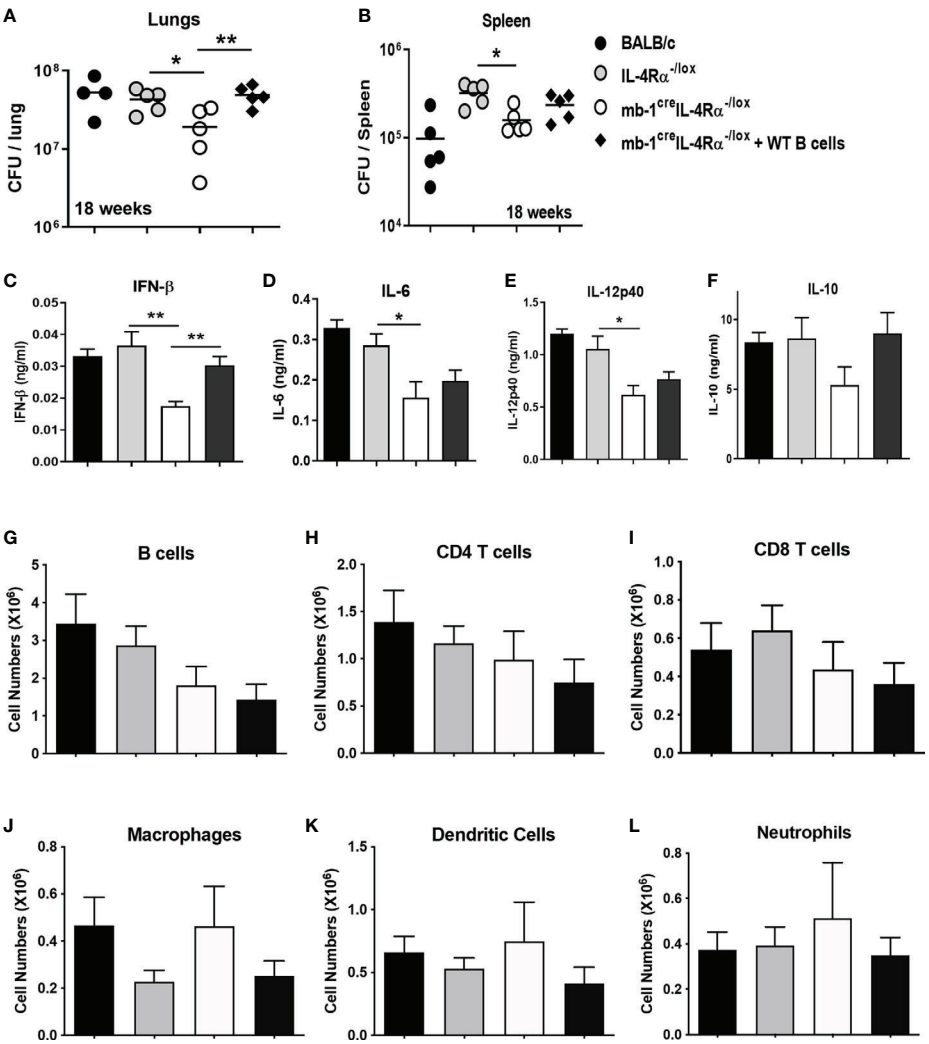

FIGURE 2 | Adoptive transfer of WT B cells intratracheally restored the bacterial burdens in B cell-specific IL-4Rα deficient mice during *Mtb* infection. Wild-type (BALB/c), littermate controls (IL-4Rα$^{-/lox}$), B cell-specific IL-4Rα deficient mice (mb1^{cre}IL-4Rα$^{-/lox}$) and adoptively transferred B cells in mb1^{cre}IL-4Rα$^{-/lox}$ mice (mb1^{cre}IL-4Rα$^{-/lox}$ + WT B cells) were infected intranasally with a 375CFU of H37Rv. **(A, B)** Bacterial burdens in the lungs and spleen after 18 weeks post-infection. **(C–F)** Lung homogenates were analysed for the cytokine responses such as for **(C)** IFN-β, **(D)** IL-6, **(E)** IL-12p40 and **(F)** IL-10 cytokine production by ELISA. Single cell suspension of lung cells was analysed for lymphoid **(G–I)** and myeloid **(J–L)** immune cell populations by flow cytometry. Cells were identified using the markers in parathesis; B cells (CD19$^+$CD3$^-$), CD4 T cells (CD3$^+$CD4$^+$), CD8 T cells (CD3$^+$CD8$^+$), dendritic cells (CD11c$^+$CD11b$^-$MHCII$^+$), macrophages (CD11b$^+$CD11c$^-$MHCII$^+$) and neutrophils (SiglecF$^-$CD11c$^-$Gr1$^+$). Data are shown as mean ± SEM of n = 5 mice/group, representative of two independent experiments, analysed by unpaired, student's t-test versus littermate control, *p < 0.05 and **p < 0.01.

mb1^{cre}IL-4Rα$^{-/lox}$ mice. Given the significant differences in IFN-β, we assessed whether *Mtb* exposure of wild type B cells influences ifnb1 mRNA expression levels. We found no difference in ifnb1 mRNA expression in *Mtb*-exposed B cells when compared to naïve cells (**Supplementary Figure 1A**). Furthermore, we flow-sorted lung B cells from chronic *Mtb*-infected mb1^{cre}IL-4Rα$^{-/lox}$ mice, which showed no difference in ifnb1, il10 and il6 mRNA expression when compared to B cells from control animals (**Supplementary Figure 1B**). These findings indicated that B cells indirectly regulate IFN-β production. IFN-β can regulate anti-inflammatory responses by inducing IL-10 expression in the context of LPS stimulated and *Mtb* infected macrophages (32, 33). However, intracellular cytokine staining revealed IL-10-producing B cells were unaffected in chronic *Mtb*-infected mb1^{cre}IL-4Rα$^{-/lox}$ mice (**Supplementary Figure 1C**). We then assessed lymphoid and myeloid immune cell populations in the lungs by flow cytometry. We found no difference in B cells (**Figure 2G**), CD4 (**Figure 2H**), CD8 T cells (**Figure 2I**), macrophages (**Figure 2J**), dendritic cells (**Figure 2K**) and neutrophils (**Figure 2L**) in the lungs of mb1^{cre}IL-4Rα$^{-/lox}$ mice. Moreover, we further analysed the B cell subsets in the lungs of *Mtb*-infected mice. We found that except CD43^{-}IgM^{+} B cells, deletion of IL-4Rα had no effect on B-1a, B-1b, B-2, B-10, plasma cells, plasmablast and IgD^{+} B cell populations when compared to control animals (**Supplementary Figures 1D, E**). We then assessed the impact of adoptively transferred wild-type B cells on lung pathology (H&E) and iNOS expression. Indeed, wild-type B cells restored lung pathology (**Figure 3A**) and lesion area (**Figure 3B**) similar to control animals but iNOS expression was unchanged (**Figures 3A, B**) in mb1^{cre}IL-4Rα$^{-/lox}$ mice. This indicates that B cells do contribute to the lung pathology independent of iNOS expression. Together, these results suggest that intact IL-4Rα on B cells contribute to mycobacterial burdens with lung pathology with no major impact on B cell subsets in chronic tuberculosis infection.

IL-4Rα Deletion Modulates Antibody Production in the Lungs of mb1^{cre}IL-4Rα$^{-/lox}$ Mice

We then explored the influence of IL-4Rα deletion on antibody responses in the lungs during *Mtb* infection. At 18 weeks post-infection, we found increased protective IgA (**Figure 3C**) in the lung homogenates. The quantification of IgA positive areas in lung sections by immunohistochemistry further confirmed our findings of IgA in the lung homogenates (**Supplementary Figure 1G**). Permissive IgG1 (**Figure 3D**) production was decreased and total IgE (**Figure 3E**) remained unaffected in the lungs of mb1^{cre}IL-4Rα$^{-/lox}$ mice when compared to littermate control animals. IL-4 is the first identified stimuli that induce IgG1 production through isotype class switching by germ-line transcript induction (34). Sterile transcripts of IgG1 in B cells stimulated with LPS and IL-4 showed a trend of decreased production germline IgG1 transcripts in splenic B cells isolated from mb1^{cre}IL-4Rα$^{-/lox}$ mice (**Supplementary Figure 1F**). Moreover, adoptively transferred B cells had no major impact on the antibodies in the lungs when compared to mb1^{cre}IL-4Rα$^{-/lox}$ mice, except IgG1 levels which interestingly further decreased (**Figure 3D**). These results suggest that IL-4Rα signalling does modulate B cell antibody responses in the lungs. To better understand the B cell responses at the molecular level, we sorted B cells from the lungs of *Mtb*-infected mice to perform quantitative PCR after 18 weeks of infection. B cells showed reduced mRNA transcripts of *tnf* and *stat1* in mb1^{cre}IL-4Rα$^{-/lox}$ mice, which was increased similar to control animals (**Figure 3F**) following adoptive transfer of B cells. This points towards a reduced Be1 signature in mb1^{cre}IL-4Rα$^{-/lox}$ mice. Despite decreased levels of *stat1* mRNA expression in B cells, we found similar levels of IFN-γ in the lungs (data not shown), suggesting that T cells and NK cells may contribute to the production of IFN-γ. Together, these results suggest that the deletion of IL-4Rα on B cells modulates lung antibody responses and decrease *tnf* and *stat1* mRNA expression in mb1^{cre}IL-4Rα$^{-/lox}$ mice during chronic tuberculosis infection.

Deletion of IL-4Rα on B Cells Reduced Association With *Mtb*

We further investigated whether B cells increase IL-4Rα expression upon *Mtb* infection *ex vivo*. Magnetic bead-sorted wild-type B cells showed that *Mtb* infection significantly increased IL-4Rα expression when compared to naïve B cells after 24 hours (**Figure 4A**), which was further confirmed by qPCR (**Supplementary Figure 1H**). We then asked whether *Mtb* may associate differentially with B cells from IL-4Rα$^{-/lox}$ and mb1^{cre}IL-4Rα$^{-/lox}$ mice. Indeed, flow cytometry revealed the frequency of mCherry-expressing *Mtb* positive cells was reduced in B cells from mb1^{cre}IL-4Rα$^{-/lox}$ mice after 24 hours (**Figure 4B**). Furthermore, we found increased MHCII positive B cells (**Figure 4C**) and expression (**Figure 4D**), indicating increased antigen presentation by the B cells from mb1^{cre}IL-4Rα$^{-/lox}$ mice when compared to controls. We then assessed the potential impact of *Mtb*-infected B cell supernatants on macrophages during infection. Following *Mtb* infection, macrophages were cultured with supernatants from *Mtb* exposed B cells from either mb1^{cre}IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/lox}$ mice. After three days, macrophages cultured with B cell supernatants from mb1^{cre}IL-4Rα$^{-/lox}$ mice showed a significant increase in nitric oxide (**Figure 4E**), IL-1β (**Figure 4F**), IL-6 (**Figure 4G**) and TNF (**Figure 4H**) production. Interestingly, IL-4Rα expression in B cells sorted from peripheral blood of TB patients at the diagnosis showed no difference in IL4R transcripts (**Figure 4I**) when compared to healthy controls. However, arginase 1 significantly decreased in blood B cells of TB patients (**Figure 4J**). Altogether, this indicate that IL-4Rα deletion on B cells does increase macrophage proinflammatory responses and their killing effector function.

DISCUSSION

Cytokine measurements in patients with TB suggested a role for IL-4Rα-driven T helper 2 immunity in the progression of the

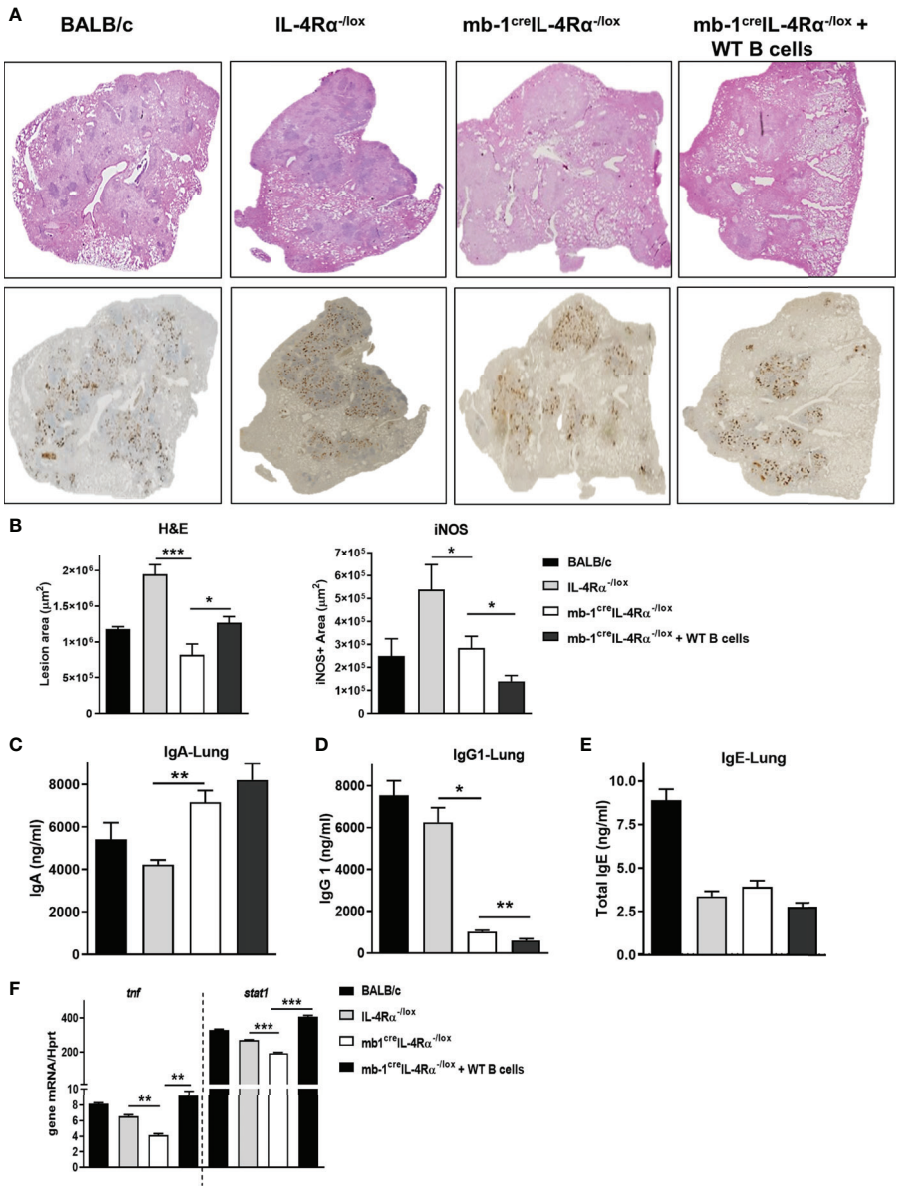

FIGURE 3 | Adoptive transfer of WT B cells in the lungs restored the pulmonary pathology in B cell-specific IL-4Rα deficient mice during *Mtb* infection. Formalin-fixed lung samples were stained for the H&E and iNOS expression after 18 weeks post-infection. **(A)** Representative histology images from all the groups and **(B)** quantification of lesions area and iNOS positive staining in the lungs (Original magnification: 2X). Antibody responses in the lungs of mice. **(C)** IgA, **(D)** IgG1 and **(E)** total IgE production in the lungs after 18 weeks of infection. **(F)** *Tnf* and *Stat1* mRNA expression in flow-sorted B cells ($CD3^-CD19^+B220^+$) after 18 weeks of *Mtb* infection. Data are shown as mean ± SEM of n = 5 mice/group, representative of two independent experiments, analysed by unpaired, student t-test versus littermate control, *p < 0.05, **p < 0.01 and ***p < 0.001.

disease (35). IL-4 secretion in PBMC is elevated and involved in cavitary granuloma formation in patients with active TB disease (36, 37). Murine models using IL-$4^{-/-}$, IL-$4R\alpha^{-/-}$ and $STAT6^{-/-}$ on genetically resistant C57BL/6 background proved to be dispensable in *Mtb* infection (21, 38, 39). Interestingly in C57BL/6 mice, transgenic expression of IL-13 uncovered that IL-13/IL-4Rα signaling contributes to TB-associated pathology (40). In contrast to overall C57BL/6, TB disease progression likely associated with Th2 immune response in BALB/c mice during chronic infection (41). Furthermore, immunotherapy using anti-IL-4 or anti-IL-13 or combined IL-4/IL-13 neutralizing antibodies (42) and high-dose *Mtb*-infected IL-$4^{-/-}$ BALB/c mice resulted in decreased bacterial loads (39) and attenuated lung pathology (41). Together, data derived from BALB/c mice demonstrated that Th2 immune response contributes to disease progression, and therefore blocking IL-4 seems an attractive therapeutic approach (43). BALB/c mice appear to be a suitable model for investigating Th2 immunity in

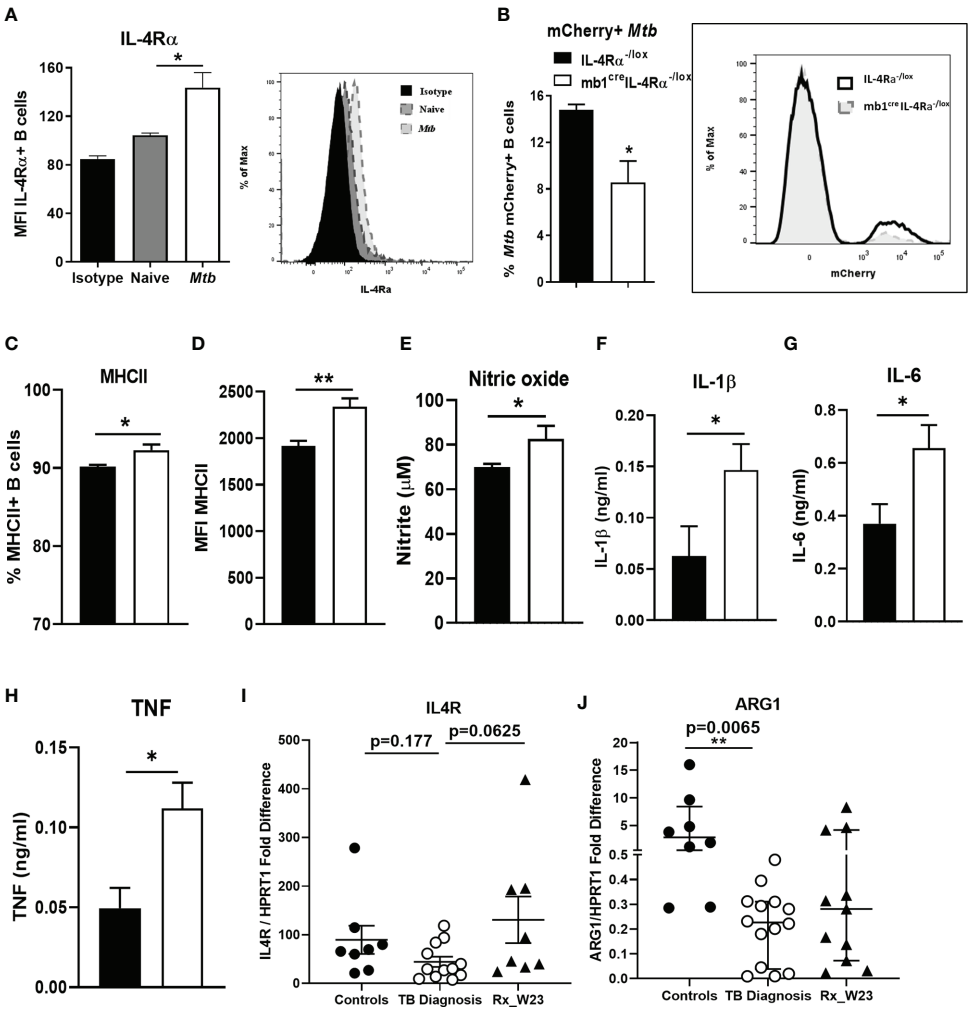

FIGURE 4 | *Mtb* infection of B cells from mb1^{cre}IL-4Rα$^{-/lox}$ mice induces pro-inflammatory responses in macrophages. B cells were purified by magnetic bead sorting from the spleen of naïve wild-type mice. B cells were then infected with H37Rv *Mtb* (MOI=2) for 24 hours. **(A)** IL-4Rα surface expression measured in naïve and *Mtb* exposed B cells by flow cytometry. Magnetic bead sorted B cells from IL-4Rα$^{-/lox}$ and mb1^{cre}IL-4Rα$^{-/lox}$ mice were infected with mCherry-expressing *Mtb* for 24 hours. **(B)** mCherry-expressing *Mtb* and **(C, D)** MHC II expression on B cells from IL-4Rα$^{-/lox}$ and mb1^{cre}IL-4Rα$^{-/lox}$ mice were analysed by flow cytometry. **(E)** *Mtb*-infected macrophages were cultured with the supernatants from the *Mtb* infected B cells for 72 hours. Supernatants were then analysed for the production of **(E)** nitric oxide, **(F)** IL-1β **(G)** IL-6 and **(H)** TNF by ELISA. Data are shown as mean ± SEM of n = 3 mice/group and representative of three independent experiments, analysed by unpaired, student t-test versus littermate control, *p < 0.05, **p < 0.01. **(I)** Human IL4R mRNA and **(J)** Arginase 1 expression was determined in magnetic bead sorted B cells from healthy, TB patients at diagnosis and after 23 weeks of anti-TB therapy by qPCR with p values between the indicated groups (n=8-12 samples) analyzed by one-way ANOVA, **p < 0.01 versus controls.

tuberculosis (44). Thus, we assessed whether depletion of IL-4Rα on B cells in mb1^{cre}IL-4Rα$^{-/lox}$ BALB/c mice in tuberculosis.

In addition to IL-4/IL-4Rα axis, B cells are critical in antibody production and they are efficient antigen-presenting cells. The success of antibodies in passive immunization suggested that certain antibodies are protective against TB (45). Mice lacking B cells showed relatively modest disease phenotypes during *Mtb* infection (19, 20). Moreover, B cell-deficient (IgH-6$^{-/-}$) mice on a C57BL/6J background were dispensable in chronic tuberculosis (21). In acute tuberculosis, B cell-mediated humoral immunity is required to control inflammation and protective immunity (19, 22, 46). Surprisingly, B cell-deficient uMT$^{-/-}$ mice infected with CDC1551 (22) strain of *Mtb* displayed similar burdens in the acute phase and reduced lung inflammation in chronic TB. In contrast, uMT$^{-/-}$ mice showed enhanced mortality as a result of increased neutrophils and IL-10 production in the lungs in the Erdman strain of *Mtb* infection (19). Moreover, the non-human primate model of cynomolgus macaques showed that *Mtb*-containing granulomas are surrounded by proliferating B cells, secreting *Mtb*-specific (IgG) antibodies (15). However, B cell depletion using rituximab resulted in highly heterogeneous responses in local granuloma immune modulation, due to the antibody-dependent and -independent functions of B cells and altogether had no impact on the TB disease outcome (25).

These studies highlighted the intricate role of B cells in TB disease stage and regulate the lung granulomatous response.

In wild-type mice, *Mtb* infection increased IL-4Rα expression on B cells and the absence of IL-4Rα on B cells decreased the frequency of *Mtb* infected B cells from mb1^{cre}IL-4Rα$^{-/lox}$ mice. There are limited studies on B cell internalization of bacteria; it has been shown that the human Raji B cell line can phagocytose complement opsonized *Mtb* (47). Macropinocytosis can also be employed by immortalized B cells for the uptake of *Mtb* (48). The effects of IL-4 signalling on phagocytosis are debatable as both increased and diminished phagocytic capacity were observed in macrophages (49–51). In the absence of IL-4Rα, we observed decreased internalization of *Mtb* by B cells; however, it warrants further studies on whether IL-4 alters phagocytic capacity and phagosome phenotype in B cells (52). Remarkably, genetic ablation of IL-4Rα on B cells (mb-1^{cre}IL-4Rα$^{-/lox}$) in mice showed reduced lung burdens and splenic dissemination in chronic tuberculosis infection. This was also accompanied by reduced lung pathology, lesion area and iNOS expression. In contrast, IL-4Rα deleted on macrophage/neutrophils, LysM^{cre}IL-4Rα$^{-/lox}$ mice had no differences in tissue bacterial burdens (29). Interestingly, the immune cell populations remained unaffected in mb1^{cre}IL-4Rα$^{-/lox}$ mice, which corroborated with our previous findings in LysM^{cre}IL-4Rα$^{-/lox}$ mice during tuberculosis (29). Characterization of lung B cell subsets also revealed no major differences between mb-1^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls except IgM^{+}IgD^{-}CD43^{-} subset. These cells are either lung B1 cells expressing low levels of CD43 or distinct anergic, short-lived, B cell receptor unresponsive cells B2 cells (53, 54). In-depth phenotyping of this subset may explain whether IL-Rα signalling is important for the maintenance and the decreased numbers of these cells contribute to protection in mb-1^{cre}IL-4Rα$^{-/lox}$ mice. In contrast, B cell depletion (rituximab) in macaques, lead to increased T cell frequencies and cytokine responses unable to drive host protection during *Mtb* infection (25). These data suggest that IL-4Rα signalling on B cells modulate *Mtb* infection more at the site of infection in the chronic phase of tuberculosis.

Remarkably, the adoptive transfer of wild-type B cells in mb-1^{cre}IL-4Rα$^{-/lox}$ mice reversed lung bacterial burdens, lung pathology and lesion area similar to wild-type mice. The absence of B cells does not affect lung IFN-γ levels (19). This is likely compensated due to the release of IFN-γ from natural killer and T cells. In tuberculosis, increased levels of type I IFN is host detrimental (55). The decreased IFN-β production in mb-1^{cre}IL-4Rα$^{-/lox}$ mice in this study might be associated with reduced tissue pathology and lung bacterial burdens. These parameters were restored upon the adoptive transfer of wild-type B cells, suggesting intact IL-4Rα on B cells enhances or mediate disease pathology, independent of B cell-mediated IFN-β production. A recent study showed that *Mtb*-stimulated IL-4Rα-sufficient B cells drive alternative activation of macrophages through IFN-β production (56). However, the absence of IL4-Rα signalling on B cells does not seem to affect IFN-β levels on B cells in both *ex vivo Mtb* exposure or *in vivo* chronic *Mtb* infection. Therefore, it is plausible that IL-4Rα-deficient B cells will hinder alternative activation macrophage phenotype through other soluble factors. Indeed, deficiency of IL-4Rα on B cells increased macrophage ability to increase proinflammatory cytokines and nitric oxide production, indicating that IL-4Rα signalling on B cells modulate macrophage immune responses during *Mtb* infection. These animals also showed decreased lung IgG1 (host detrimental) and increased lung IgA (host protective) levels, which may partly contribute to protection against *Mtb* infection. Besides tuberculosis, mb-1^{cre}IL-4Rα$^{-/lox}$ mice during *N. brasiliensis* infection uncovered that IL-4Rα-responsive B cells-driven IL-13 and antigen processing contribute to T cell-mediated protective immunity (13). Furthermore, we demonstrated that IL-4Rα-responsive B cells are host detrimental against *Leishmania major* and host protective in *Schistosoma mansonii* infection. Mechanistically, we revealed a more general phenomenon that B cells regulate T cell polarization (10). Moreover, in *S. mansonii* infection, IL-4Rα-expressing B cells reduced egg-driven host detrimental tissue granulomatous inflammation *via* host protective IL-10 production in mice (57). In contrast, we found neither IL-10 nor evident regulation of T cell responses rather macrophage response modulation in mb1^{cre}IL-4Rα$^{-/lox}$ mouse model in TB, indicating the underlying mechanism is different and appears more local at the site of disease.

B cell proliferation increased in latent TB granuloma and decreased in an active TB granuloma. In humans, a study showed lower IL-4 expression in human B cells in circulation during TB infection (58). Consistently, we observed a lower trend in IL-4R and a significant decrease in arginase 1 mRNA expression in peripheral blood human B cells sorted from individuals diagnosed with TB pointing towards Be1 phenotype in this cohort. These findings further reinforce that blood may not be an appropriate compartment to explore the local tissue effect of B cells (56, 59). The importance of tissue site is further demonstrated where IFN-β production was unaffected in B cells isolated from peripheral blood but significantly upregulated in B cells from the pleural fluid (56). Therefore, B cells isolated from the lungs of TB patients will increase our current understanding of immune modulation at the tissue level. Overall, our study reveals the underappreciated role of IL-4Rα signalling on B cells during the chronic phase of tuberculosis infection in mice.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Participant recruitment and follow-up were approved by the Human Research Ethics Committee of Stellenbosch University (N10/01/013). Written informed consent was obtained from all

study participants. The patients/participants provided their written informed consent to participate in this study. The protocol was approved by the Animal Ethics Committee (AEC Permit Number: 015/040), Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa.

AUTHOR CONTRIBUTIONS

SP, MO, IR, and AL: designing research studies, conducting experiments, human samples and analysis. SP, MO, MH, JC, RG, and RK: acquiring data and analyzing data. SP and MO: writing the manuscript. FB: resources and funding for the research. All authors contributed to the article and approved the submitted version.

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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.2021.611673/full#supplementary-material

Supplementary Figure 1 | Deletion of IL-4Rα on B cells had no impact on cytokine genes expression and B cell subsets. **(A)** Magnetic-bead sorted B cells from the spleen of naïve mice were exposed to *Mtb* (MOI=2) for 24 hours. *Ifnb* mRNA expression was determined by qPCR. Wild-type (BALB/c), littermate controls (IL-4Rα$^{-/lox}$) and B cell-specific IL-4Rα deficient mice (mb-1^{cre}IL-4Rα$^{-/lox}$) were infected intranaslly with a dose of 150 CFU H37Rv. **(B)** Flow-sorted B cells (CD19^{+}B220^{+}CD3^{-}) were analysed for *Ifnb1, il10* and *il-6* transcripts by qPCR. **(C)** The frequency of IL-10-producing B cells after 10 hours of PMA (20ng/ml)/ionomycin (1μg/ml) stimulation was determined by intracellular cytokine assay after 12 weeks post-infection. **(D)** B-1a (CD19^{+}B220^{+}CD43^{+}CD5^{high}IgM^{high}), B1b (CD19^{+}B220^{+}CD43^{+}CD5^{low}IgM^{high}), B2 (CD19^{+}B220^{+}CD43^{-}IgM^{+}IgD^{+}) B10 (CD19^{+}B220^{+}CD43^{+}CD5^{+}CD1d^{+}) and IgM (CD19^{+}B220^{+}CD43^{-}IgM^{+}IgD^{-}). **(E)** Plasma (CD19^{+}B220^{+} CD138^{+}MHCII^{low}CD44^{high}), Plasmablast (CD19^{+}B220^{+}CD138^{+}MHCII^{high}CD44^{high}) and IgD (CD19^{+}B220^{+}IgD^{+}) B cells in the lungs of mice after 12 weeks of *Mtb* infection. **(F)** Germline transcript of *IgG1* measured by qPCR in splenic B cells stimulated with LPS (10ug/ml)/IL-4 (25ng/ml) for 48 hours. **(G)** Representative images and quantification of IgA-positive area of lung sections at 18 weeks post-infection (Original magnification: 40X). **(H)** B cells either left alone or infected with *Mtb* to determine IL-4Rα mRNA expression by qPCR. Data are shown as mean ± SEM of n = 6 mice/group, analysed by unpaired, student t-test or ordinary one-way ANOVA versus the indicated group, *p<0.05, **p<0.01 and ***p<0.001.

Supplementary Figure 2 | Gating strategy for B cell subset identification in chronic *Mtb*-infected lungs of mice.

Supplementary Figure 3 | Gating strategy for various immune cell populations in chronic *Mtb*-infected lungs of mice.

Supplementary Table 1 | The list of antibody fluorophores and clones.

Supplementary Table 2 | The list of primer sequences used for qPCR.

REFERENCES

1. Lund FE. Cytokine-Producing B Lymphocytes-Key Regulators of Immunity. *Curr Opin Immunol* (2008) 20(3):332–8. doi: 10.1016/j.coi.2008.03.003
2. du Plessis WJ, Kleynhans L, du Plessis N, Stanley K, Malherbe ST, Maasdorp E, et al. The Functional Response of B Cells to Antigenic Stimulation: A Preliminary Report of Latent Tuberculosis. *PloS One* (2016) 11(4):e0152710. doi: 10.1371/journal.pone.0152710
3. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, et al. Reciprocal Regulation of Polarized Cytokine Production by Effector B and T Cells. *Nat Immunol* (2000) 1(6):475–82. doi: 10.1038/82717
4. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic Intestinal Inflammatory Condition Generates IL-10-Producing Regulatory B Cell Subset Characterized by CD1d Upregulation. *Immunity* (2002) 16(2):219–30. doi: 10.1016/S1074-7613(02)00274-1
5. Mauri C, Gray D, Mushtaq N, Londei M. Prevention of Arthritis by Interleukin 10-Producing B Cells. *J Exp Med* (2003) 197(4):489–501. doi: 10.1084/jem.20021293
6. Singh A, Carson WFT, Secor ERJr., Guernsey LA, Flavell RA, Clark RB, et al. Regulatory Role of B Cells in a Murine Model of Allergic Airway Disease. *J Immunol* (2008) 180(11):7318–26. doi: 10.4049/jimmunol.180.11.7318
7. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B Cells Prevent and Reverse Allergic Airway Inflammation Via Foxp3-Positive T Regulatory Cells in a Murine Model. *J Allergy Clin Immunol* (2010) 125(5):1114–24.e8. doi: 10.1016/j.jaci.2010.01.018
8. van Rensburg IC, Kleynhans L, Keyser A, Walzl G, Loxton AG. B-Cells With a FasL Expressing Regulatory Phenotype Are Induced Following Successful Anti-Tuberculosis Treatment. *Immun Inflammation Dis* (2017) 5(1):57–67. doi: 10.1002/iid3.140
9. Harris DP, Goodrich S, Gerth AJ, Peng SL, Lund FE. Regulation of IFN-Gamma Production by B Effector 1 Cells: Essential Roles for T-Bet and the IFN-Gamma Receptor. *J Immunol* (2005) 174(11):6781–90. doi: 10.4049/jimmunol.174.11.6781
10. Hurdayal R, Ndlovu HH, Revaz-Breton M, Parihar SP, Nono JK, Govender M, et al. IL-4-Producing B Cells Regulate T Helper Cell Dichotomy in Type 1- and Type 2-Controlled Diseases. *Proc Natl Acad Sci USA* (2017) 114(40):E8430–E9. doi: 10.1073/pnas.1708125114
11. Harris DP, Goodrich S, Mohrs K, Mohrs M, Lund FE. Cutting Edge: The Development of IL-4-Producing B Cells (B Effector 2 Cells) Is Controlled by IL-4, IL-4 Receptor Alpha, and Th2 Cells. *J Immunol* (2005) 175(11):7103–7. doi: 10.4049/jimmunol.175.11.7103

12. Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, Kusser K, et al. Cytokine-Producing Effector B Cells Regulate Type 2 Immunity to H. Polygyrus. *Immunity* (2009) 30(3):421–33. doi: 10.1016/j.immuni.2009.01.006
13. Horsnell WG, Darby MG, Hoving JC, Nieuwenhuizen N, McSorley HJ, Ndlovu H, et al. Il-4Ralpha-Associated Antigen Processing by B Cells Promotes Immunity in Nippostrongylus Brasiliensis Infection. *PloS Pathog* (2013) 9(10):e1003662. doi: 10.1371/journal.ppat.1003662
14. Tsai MC, Chakravarty S, Zhu G, Xu J, Tanaka K, Koch C, et al. Characterization of the Tuberculous Granuloma in Murine and Human Lungs: Cellular Composition and Relative Tissue Oxygen Tension. *Cell Microbiol* (2006) 8(2):218–32. doi: 10.1111/j.1462-5822.2005.00612.x
15. Phuah JY, Mattila JT, Lin PL, Flynn JL. Activated B Cells in the Granulomas of Nonhuman Primates Infected With Mycobacterium Tuberculosis. *Am J Pathol* (2012) 181(2):508–14. doi: 10.1016/j.ajpath.2012.05.009
16. Ulrichs T, Kosmiadi GA, Jorg S, Pradl L, Titukhina M, Mishenko V, et al. Differential Organization of the Local Immune Response in Patients With Active Cavitary Tuberculosis or With Nonprogressive Tuberculoma. *J Infect Dis* (2005) 192(1):89–97. doi: 10.1086/430621
17. Ulrichs T, Kosmiadi GA, Trusov V, Jorg S, Pradl L, Titukhina M, et al. Human Tuberculous Granulomas Induce Peripheral Lymphoid Follicle-Like Structures to Orchestrate Local Host Defence in the Lung. *J Pathol* (2004) 204(2):217–28. doi: 10.1002/path.1628
18. Clatworthy MR. Targeting B Cells and Antibody in Transplantation. *Am J Transplant* (2011) 11(7):1359–67. doi: 10.1111/j.1600-6143.2011.03554.x
19. Maglione PJ, Xu J, Chan J. B Cells Moderate Inflammatory Progression and Enhance Bacterial Containment Upon Pulmonary Challenge With Mycobacterium Tuberculosis. *J Immunol* (2007) 178(11):7222–34. doi: 10.4049/jimmunol.178.11.7222
20. Vordermeier HM, Venkataprasad N, Harris DP, Ivanyi J. Increase of Tuberculous Infection in the Organs of B Cell-Deficient Mice. *Clin Exp Immunol* (1996) 106(2):312–6. doi: 10.1046/j.1365-2249.1996.d01-845.x
21. Turner J, Frank AA, Brooks JV, Gonzalez-Juarrero M, Orme IM. The Progression of Chronic Tuberculosis in the Mouse Does Not Require the Participation of B Lymphocytes or Interleukin-4. *Exp Gerontol* (2001) 36 (3):537–45. doi: 10.1016/S0531-5565(00)00257-6
22. Bosio CM, Gardner D, Elkins KL. Infection of B Cell-Deficient Mice With CDC 1551, A Clinical Isolate of Mycobacterium Tuberculosis: Delay In Dissemination and Development of Lung Pathology. *J Immunol* (2000) 164 (12):6417–25. doi: 10.4049/jimmunol.164.12.6417
23. Kozakiewicz L, Chen Y, Xu J, Wang Y, Dunussi-Joannopoulos K, Ou Q, et al. B Cells Regulate Neutrophilia During Mycobacterium Tuberculosis Infection and BCG Vaccination by Modulating the interleukin-17 Response. *PloS Pathog* (2013) 9(7):e1003472. doi: 10.1371/journal.ppat.1003472
24. Torrado E, Fountain JJ, Robinson RT, Martino CA, Pearl JE, Rangel-Moreno J, et al. Differential and Site Specific Impact of B Cells in the Protective Immune Response to Mycobacterium Tuberculosis in the Mouse. *PloS One* (2013) 8(4):e61681. doi: 10.1371/journal.pone.0061681
25. Phuah J, Wong EA, Gideon HP, Maiello P, Coleman MT, Hendricks MR, et al. Effects of B Cell Depletion on Early Mycobacterium Tuberculosis Infection in Cynomolgus Macaques. *Infect Immun* (2016) 84(5):1301–11. doi: 10.1128/IAI.00083-16
26. Nolan A, Fajardo E, Huie ML, Condos R, Pooran A, Dawson R, et al. Increased Production of IL-4 and IL-12p40 From Bronchoalveolar Lavage Cells Are Biomarkers of Mycobacterium Tuberculosis in the Sputum. *PloS One* (2013) 8(3):e59461. doi: 10.1371/journal.pone.0059461
27. Hussain R, Talat N, Ansari A, Shahid F, Hasan Z, Dawood G. Endogenously Activated Interleukin-4 Differentiates Disease Progressors and Non-Progressors in Tuberculosis Susceptible Families: A 2-Year Biomarkers Follow-Up Study. *J Clin Immunol* (2011) 31(5):913–23. doi: 10.1007/s10875-011-9566-y
28. Ordway DJ, Costa L, Martins M, Silveira H, Amaral L, Arroz MJ, et al. Increased Interleukin-4 Production by CD8 and Gammadelta T Cells in Health-Care Workers Is Associated With the Subsequent Development of Active Tuberculosis. *J Infect Dis* (2004) 190(4):756–66. doi: 10.1086/422532
29. Guler R, Parihar SP, Savvi S, Logan E, Schwegmann A, Roy S, et al. Il-4Ralpha-dependent Alternative Activation of Macrophages Is Not Decisive for Mycobacterium Tuberculosis Pathology and Bacterial Burden in Mice. *PloS One* (2015) 10(3):e0121070. doi: 10.1371/journal.pone.0121070
30. Pooran A, Davids M, Nel A, Shoko A, Blackburn J, Dheda K. IL-4 Subverts Mycobacterial Containment in Mycobacterium Tuberculosis-Infected Human Macrophages. *Eur Respir J* (2019) 54(2):1802242 doi: 10.1183/13993003.02242-2018
31. Hoving JC, Kirstein F, Nieuwenhuizen NE, Fick LC, Hobeika E, Reth M, et al. B Cells That Produce Immunoglobulin E Mediate Colitis in BALB/c Mice. *Gastroenterology* (2012) 142(1):96–108. doi: 10.1053/j.gastro.2011.09.044
32. McNab FW, Ewbank J, Howes A, Moreira-Teixeira L, Martirosyan A, Ghilardi N, et al. Type I IFN Induces IL-10 Production in an IL-27-independent Manner and Blocks Responsiveness to IFN-Gamma for Production of IL-12 and Bacterial Killing in Mycobacterium Tuberculosis-Infected Macrophages. *J Immunol* (2014) 193(7):3600–12. doi: 10.4049/jimmunol.1401088
33. Chang EY, Guo B, Doyle SE, Cheng G. Cutting Edge: Involvement of the Type I IFN Production and Signaling Pathway in Lipopolysaccharide-Induced IL-10 Production. *J Immunol* (2007) 178(11):6705–9. doi: 10.4049/jimmunol.178.11.6705
34. Lutzker S, Rothman P, Pollock R, Coffman R, Alt FW. Mitogen- and IL-4-regulated Expression of Germ-Line Ig Gamma 2b Transcripts: Evidence for Directed Heavy Chain Class Switching. *Cell* (1988) 53(2):177–84. doi: 10.1016/0092-8674(88)90379-0
35. Rook GA. Th2 Cytokines in Susceptibility to Tuberculosis. *Curr Mol Med* (2007) 7(3):327–37. doi: 10.2174/156652407780598557
36. Mazzarella G, Bianco A, Perna F, D'Auria D, Grella E, Moscariello E, et al. T Lymphocyte Phenotypic Profile in Lung Segments Affected by Cavitary and Non-Cavitary Tuberculosis. *Clin Exp Immunol* (2003) 132(2):283–8. doi: 10.1046/j.1365-2249.2003.02121.x
37. Seah GT, Scott GM, Rook GA. Type 2 Cytokine Gene Activation and its Relationship to Extent of Disease in Patients With Tuberculosis. *J Infect Dis* (2000) 181(1):385–9. doi: 10.1086/315200
38. Jung YJ, LaCourse R, Ryan L, North RJ. Evidence Inconsistent With a Negative Influence of T Helper 2 Cells on Protection Afforded by a Dominant T Helper 1 Response Against Mycobacterium Tuberculosis Lung Infection in Mice. *Infect Immun* (2002) 70(11):6436–43. doi: 10.1128/IAI.70.11.6436-6443.2002
39. North RJ. Mice Incapable of Making IL-4 or IL-10 Display Normal Resistance to Infection With Mycobacterium Tuberculosis. *Clin Exp Immunol* (1998) 113 (1):55–8. doi: 10.1046/j.1365-2249.1998.00636.x
40. Heitmann L, Abad Dar M, Schreiber T, Erdmann H, Behrends J, McKenzie AN, et al. The IL-13/IL-4Ralpha Axis Is Involved in Tuberculosis-Associated Pathology. *J Pathol* (2014) 234(3):338–50. doi: 10.1002/path.4399
41. Hernandez-Pando R, Aguilar D, Hernandez ML, Orozco H, Rook G. Pulmonary Tuberculosis in BALB/c Mice With non-Functional IL-4 Genes: Changes in the Inflammatory Effects of TNF-Alpha and in the Regulation of Fibrosis. *Eur J Immunol* (2004) 34(1):174–83. doi: 10.1002/eji.200324253
42. Roy E, Brennan J, Jolles S, Lowrie DB. Beneficial Effect of Anti-Interleukin-4 Antibody When Administered in a Murine Model of Tuberculosis Infection. *Tuberculosis (Edinb)* (2008) 88(3):197–202. doi: 10.1016/j.tube.2007.11.005
43. Hernandez-Pando R, Orozcoe H, Sampieri A, Pavon L, Velasquillo C, Larriva-Sahd J, et al. Correlation Between the Kinetics of Th1, Th2 Cells and Pathology in a Murine Model of Experimental Pulmonary Tuberculosis. *Immunology* (1996) 89(1):26–33.
44. Buccheri S, Reljic R, Caccamo N, Ivanyi J, Singh M, Salerno A, et al. IL-4 Depletion Enhances Host Resistance and Passive IgA Protection Against Tuberculosis Infection in BALB/c Mice. *Eur J Immunol* (2007) 37(3):729–37. doi: 10.1002/eji.200636764
45. Jacobs AJ, Mongkolsapaya J, Screaton GR, McShane H, Wilkinson RJ. Antibodies and Tuberculosis. *Tuberculosis (Edinb)* (2016) 101:102–13. doi: 10.1016/j.tube.2016.08.001
46. Maglione PJ, Chan J. How B Cells Shape the Immune Response Against Mycobacterium Tuberculosis. *Eur J Immunol* (2009) 39(3):676–86. doi: 10.1002/eji.200839148
47. Zhu Q, Zhang M, Shi M, Liu Y, Zhao Q, Wang W, et al. Human B Cells Have an Active Phagocytic Capability and Undergo Immune Activation Upon Phagocytosis of Mycobacterium Tuberculosis. *Immunobiology* (2016) 221 (4):558–67. doi: 10.1016/j.imbio.2015.12.003
48. Garcia-Perez BE, de la Cruz-Lopez JJ, Castaneda-Sanchez JI, Munoz-Duarte AR, Hernandez-Perez AD, Villegas-Castrejon H, et al. Macropinocytosis Is Responsible for the Uptake of Pathogenic and Non-Pathogenic Mycobacteria by B Lymphocytes (Raji Cells). *BMC Microbiol* (2012) 12:246. doi: 10.1186/1471-2180-12-246

49. Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M, et al. Alternative Activation Is an Innate Response to Injury That Requires CD4+ T Cells to be Sustained During Chronic Infection. *J Immunol* (2007) 179 (6):3926–36. doi: 10.4049/jimmunol.179.6.3926
50. Varin A, Mukhopadhyay S, Herbein G, Gordon S. Alternative Activation of Macrophages by IL-4 Impairs Phagocytosis of Pathogens But Potentiates Microbial-Induced Signalling and Cytokine Secretion. *Blood* (2010) 115 (2):353–62. doi: 10.1182/blood-2009-08-236711
51. Wirth JJ, Kierszenbaum F, Zlotnik A. Effects of IL-4 on Macrophage Functions: Increased Uptake and Killing of a Protozoan Parasite (Trypanosoma Cruzi). *Immunology* (1989) 66(2):296–301.
52. de Keijzer S, Meddens MB, Kilic D, Joosten B, Reinieren-Beeren I, Lidke DS, et al. Interleukin-4 Alters Early Phagosome Phenotype by Modulating Class I PI3K Dependent Lipid Remodeling and Protein Recruitment. *PloS One* (2011) 6(7):e22328. doi: 10.1371/journal.pone.0022328
53. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 Cells in the Bone Marrow Are a Significant Source of Natural IGM. *Eur J Immunol* (2012) 42 (1):120–9. doi: 10.1002/eji.201141890
54. Nguyen TT, Elsner RA, Baumgarth N. Natural IgM Prevents Autoimmunity by Enforcing B Cell Central Tolerance Induction. *J Immunol* (2015) 194 (4):1489–502. doi: 10.4049/jimmunol.1401880
55. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The Immune Response in Tuberculosis. *Annu Rev Immunol* (2013) 31:475–527. doi: 10.1146/annurev-immunol-032712-095939
56. Benard A, Sakwa I, Schierloh P, Colom A, Mercier I, Tailleux L, et al. B Cells Producing Type I IFN Modulate Macrophage Polarization in Tuberculosis. *Am J Respir Crit Care Med* (2018) 197(6):801–13. doi: 10.1164/rccm.201707-1475OC
57. Ndlovu H, Nono JK, Abdel Aziz N, Nieuwenhuizen NE, Brombacher F. Interleukin-4 Receptor Alpha Expressing B Cells Are Essential to Down-Modulate Host Granulomatous Inflammation During Schistosomasis. *Front Immunol* (2018) 9:2928. doi: 10.3389/fimmu.2018.02928
58. van Rensburg IC, Wagman C, Stanley K, Beltran C, Ronacher K, Walzl G, et al. Successful TB Treatment Induces B-Cells Expressing FASL and IL5RA Mrna. *Oncotarget* (2017) 8(2):2037–43. doi: 10.18632/oncotarget.12184
59. Moore DK, Leisching GR, Snyders CI, Gutschmidt A, van Rensburg IC, Loxton AG. Immunoglobulin Profile and B-Cell Frequencies Are Altered With Changes in the Cellular Microenvironment Independent of the Stimulation Conditions. *Immun Inflammation Dis* (2020) 8(3):458–67. doi: 10.1002/iid3.328

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