MODULATING CYTOKINES AS TREATMENT FOR AUTOIMMUNE DISEASES AND CANCER

EDITED BY: Erwan Mortier, Averil Ma, Barbara A. Malynn and Markus Neurath
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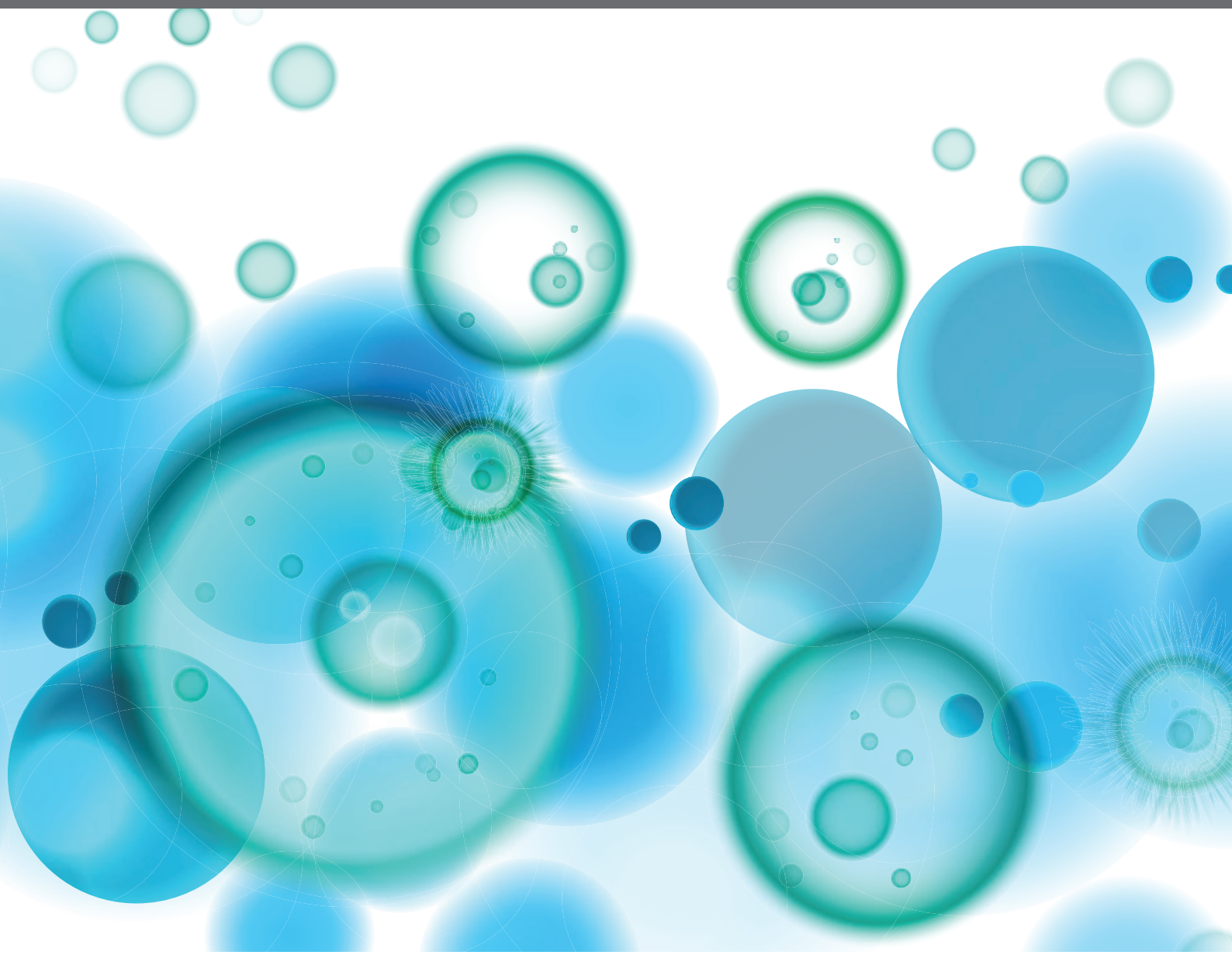

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MODULATING CYTOKINES AS TREATMENT FOR AUTOIMMUNE DISEASES AND CANCER

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EDITORIAL
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Editorial: Modulating Cytokines as Treatment for Autoimmune Diseases and Cancer

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Editorial on the Research Topic

Modulating Cytokines as Treatment for Autoimmune Diseases and Cancer

INTRODUCTION

Cytokines are key mediators in the regulation of the normal immune response. They are ambivalent molecules which can be either beneficial to the treatment of diseases, but can also be harmful and participate in pathogenesis. Indeed, despite regulatory controls at multiple levels, abnormal immune responses involving cytokines can occur and cause various pathologies, including autoimmunity and inflammation-induced cancer. For these reasons, it is crucial to continue efforts focused on understanding the different modes of action of cytokines with an eye toward the design of new selective drugs that modulate cytokine activities that direct beneficial immune responses.

Deregulation of cytokine expression has a complex role in disease pathogenesis and novel therapeutic agents that neutralize cytokines have been successfully translated into clinical practice. For instance, the use of monoclonal anti-TNF antibodies have greatly improved the health of patients suffering from diseases like inflammatory bowel diseases, rheumatoid arthritis, spondyloarthritis, or psoriasis. Furthermore, additional cytokine blockers such as anti-IL-6R antibodies, IL-12/IL-23 p40 inhibitors and IL-23 p19 blockers have been approved for various immune-mediated diseases. The use of effector cytokines (e.g., IL-2, IFNγ) either alone or in combination with other therapeutic reagents, such as checkpoint inhibitors and emerging immunocytokines, is accelerating in cancer immunotherapy. While, IL-2 was approved by the Food and Drug Administration for the treatment of metastatic kidney cancer in 1992 and for metastatic melanoma in 1998, researchers are still working to improve IL-2 efficacy and reduce toxicity. Given the broad range of biological activities of cytokines, the side effects of biologic therapies need to be carefully assessed and warrant the development of new therapeutics with improved specificity of action. Thus, fundamental discoveries on structural features of cytokines in interaction with their different receptor chains could lead to the identification of cytokines with reduced toxicity and increased specificity.

In this Research Topic issue entitled "Modulating Cytokines as Treatment of Autoimmune Diseases and Cancer", we have compiled 6 original research articles, 1 hypothesis and theory and 6 reviews. This collection is divided into four sections. The first section presents recent knowledge for a better understanding of the mode of action and the structural features of the interaction between cytokines and their receptors. The second section describes new targets for the treatment of

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autoimmune diseases. The third is devoted to the use of cytokines to induce tolerance and the last section presents different combinations between cytokines and other therapeutic agents for the treatment of cancer.

BETTER UNDERSTANDING OF CYTOKINE STRUCTURE/FUNCTION AND THEIR MODES OF ACTION

Increasing knowledge of the structural interactions between a cytokine and its receptor chains is fundamental for generating original selective reagents targeting cytokine's action. In their review, Markovic and Savvides focused on the structure and the mode of signaling assemblies of two closely related cytokines that share the IL-7Rα chain, IL-7 and TSLP. The review of Metcalfe et al. is devoted to the IL-6 family with a focus on IL-11. The authors of both reviews present structural overviews of the two cytokine systems and their involvements in pathological conditions. They also provide an overview of the broad array of potential therapeutic agents in autoimmune diseases to thwart overexpression of the targeted cytokines including monoclonal antibodies, chemical compounds, soluble receptors, and muteins. Along this line, Holgado et al. focused on an original strategy for inhibiting the action of cytokines by generating cytokine-traps. Their approach is based on the generation of molecules consisting of the fusion of receptors chains to form soluble heterodimers capable of capturing cytokines before interacting with their membrane-bound receptors. They are able to efficiently modulate either IL-33 alone by the IL-33-Trap or two cytokines simultaneously using the dual IL-14/13-Trap to inhibit experimental airway inflammation.

Cytokines are primarily described as soluble factors, but they can also be packaged within extracellular vesicles.

In their review, Barnes and Somerville provide a new vision of the action of cytokines and lead us to bear in mind that the production of both cytokines and extracellular vesicles play an important role in pathology. These properties could be translated into therapy by engineering extracellular vesicles to deliver immune modulators such as cytokines in pathological conditions.

LOOKING FOR NEW TARGETS

The research community is always on the lookout for new targets for the design of novel therapeutics. Thus, it is crucial to deepen our knowledge of relevant targets involved in pathology. Brune et al. focused on IRF5, a key transcriptional regulatory factor of type-I interferon. Hyperactivation of IRF5 has been identified as key factor in several autoimmune diseases, including systemic lupus erythematosus. In their review, Brune et al. provide an original perspective on the complex role of IRF5 and focus on T cell functions and polarization.

Innate lymphoid cells (ILC) are unique cell populations that play important roles in immune defense in response to chronic inflammatory and autoimmune diseases. Schulz-Kuhnt et al. focused on recent knowledge of the functions of human ILCs and provide a comprehensive view of the major regulators, including cytokines, that selectively support the three ILC subpopulations. A better understanding of the regulation of human ILC functions should help researchers use ILCs and modulate their action under inflammatory conditions in the future.

Adipokines are cytokines produced by adipocytes. Among them, visfatin appears to play an important role in the pathogenesis of rheumatoid arthritis (RA) by increasing the adhesion of RA synovial fibroblasts to endothelial cells. Hasseli et al. draw attention to visfatin and other adipokines as potentially interesting targets in the search for RA therapeutics.

In generating new reagents, investigators need to assess their efficacy in relevant animal models. In their study, Lio et al. analyzed the literature that used dry eye disease models to find cells and cytokines that could be targeted in this pathology. They show the involvement of Th1 cells as well as IL-1β and TNFα proinflammatory cytokines. This meta-analysis also prompts precaution when using animal models that do not fully recapitulate human pathology.

USE OF CYTOKINES TO INDUCE TOLERANCE

Autoimmune diseases are characterized by the disruption of tolerance to self antigens. Different approaches have been designed by researchers to restore tolerance, including cell therapy by injecting tolerogenic dendritic cells (Tol-DC) or regulatory T (T-reg) cells. Another approach is to target tolerogenic cells directly in vivo. Cauwels and Tavernier proposed an original strategy to expand endogenous Tol-DC in vivo by the administration of AcTakine molecules. The latter consists of a targeting module (VHH is more commonly used) fused to a mutated cytokine with reduced affinity to its cognate receptor. They engineered a Tol-DC AcTaferon with IFN-I to induce tolerance in autoimmune diseases.

IL-2 is an important cytokine for the development of T-reg cells, which constitutively express IL-2Rα. The latter forms a trimeric receptor with IL-2Rβ and the common gamma chain and binds IL-2 with a high affinity allowing T-reg cells to respond to low dose of IL-2. In their study, Ghelani et al. attempt to find the threshold required for IL-2 to selectively expand T-reg cells into effector cells. To this end, they generated a series of IL-2 muteins and found that minimal IL-2 receptor signaling is required to fully expand regulatory T cells and support their immunosuppressive functions.

IL-34 is another cytokine with tolerogenic properties. In their study, Bézie et al. show that CD4 and CD8 FoxP3 regulatory T cells increase significantly when cultured in the presence of monocytes differentiated by IL-34. In addition, human CD8 regulatory T cells grown under these conditions suppress the immune response in a humanized model of acute GVHD by effectively increasing the survival of the graft after organ transplantation by acting on T-regs cells and monocytes. These

results demonstrate that IL-34 should also be considered for therapy with regard to its property for promoting the development of regulatory T cells.

CYTOKINES IN COMBINATION WITH OTHER THERAPEUTIC AGENTS IN CANCER

IL-15 is a cytokine that shares with IL-2 an important role in supporting the development and functions of effector cells, such as NK and CD8 T cells. Unlike IL-2, IL-15 does not support regulatory T cells. For these reasons, IL-15 was identified early as a potential candidate for use in cancer immunotherapy. However, IL-15 administrated as monotherapy did not show therapeutic efficacy despite a dramatic expansion of NK and CD8 T cells. In their review, Waldmann et al. present assays that use IL-15 in combination with therapeutic monoclonal antibodies such as anti-CD40, anti-CD20, anti-CD52, anti-EGFR, anti-CTLA-4, anti-PD-1 and anti-PDL-1. Preliminary combination studies show better efficacy than individual agents alone and hold promise for the treatment of patients with metastatic malignancy.

Another way to combine cytokines and therapeutic monoclonal antibodies is to fuse them to generate immunocytokines (ICK). In their study, Shen et al. fused anti-PD-1 antibody to IL-21 muteins. The advantages of such molecules are numerous. They improve both the half-life and bioavailability of cytokines. Targeting cytokine therapies to specific cells may better replicate the paracrine activities of physiologically delivered cytokines. This approach can thus enhance efficacy and limit off-target effects. To restrict IL-21 activity on targeted cells and slow down the clearance of the ICK, the investigators mutated the cytokine to decrease its affinity for its cognate receptor. Preliminary preclinical data reveal that the anti-PD1-attenuated-IL-21 immunocytokine shows promise for anti-cancer indications.

Collectively, the above studies highlight the potential of recombinant cytokines and their inhibitors to more effectively treat autoimmunity and cancer. These results open new avenues for research and may lead to improved therapeutic options in clinical therapy by precision editing of cytokine responses.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Engineered IL-21 Cytokine Muteins Fused to Anti-PD-1 Antibodies Can Improve CD8+ T Cell Function and Anti-tumor Immunity

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Inhibitors that block the programmed cell death-1 (PD-1) pathway can potentiate endogenous antitumor immunity and have markedly improved cancer survival rates across a broad range of indications. However, these treatments work for only a minority of patients. The efficacy of anti-PD-1 inhibitors may be extended by cytokines, however, the incorporation of cytokines into therapeutic regimens has significant challenges. In their natural form when administered as recombinant proteins, cytokine treatments are often associated with low response rates. Most cytokines have a short half-life which limits their exposure and efficacy. In addition, cytokines can activate counterregulatory pathways, in the case of immune-potentiating cytokines this can lead to immune suppression and thereby diminish their potential efficacy. Improving the drug-like properties of natural cytokines using protein engineering can yield synthetic cytokines with improved bioavailability and tissue targeting, allowing for enhanced efficacy and reduced off-target effects. Using structure guided engineering we have designed a novel class of antibody-cytokine fusion proteins consisting of a PD-1 targeting antibody fused together with an interleukin-21 (IL-21) cytokine *mutein*. Our bifunctional fusion proteins can block PD-1/programmed death-ligand 1 (PD-L1) interaction whilst simultaneously delivering IL-21 cytokine to PD-1 expressing T cells. Targeted delivery of IL-21 can improve T cell function in a manner that is superior to anti-PD-1 monotherapy. Fusion of engineered IL-21 variants to anti-PD1 antibodies can improve the drug-like properties of IL-21 cytokine leading to improved cytokine serum half-life allowing for less frequent dosing. In addition, we show that targeted delivery of IL-21 can minimize any potential detrimental effect on local antigen-presenting cells. A highly attenuated IL-21 *mutein* variant (R9E:R76A) fused to a PD-1 antibody provides protection in a humanized mouse model of cancer that is refractory to anti-PD-1 monotherapy. Collectively, our preclinical data demonstrate that this approach may improve upon and extend the utility of anti-PD-1 therapeutics currently in the clinic.

Keywords: cancer, engineered cytokine, IL-21, PD-1, bifunctional fusion, immunotherapy

INTRODUCTION

Antibodies, that block T cell inhibitory receptors support superior priming and allow dysfunctional T cells to reengage and eradicate established cancers, have transformed the treatment of cancer (1, 2). Despite the success of co-inhibitory receptor antagonists these treatments work for only a small subset of patients (3). PD-1 is a cell surface co-inhibitory receptor expressed on activated T cells (1, 2, 4, 5). When engaged, PD-1 works to constrain T cell function by increasing the threshold for activation leading to diminished anti-tumor immune responses (1, 2, 4, 5). Combinatorial approaches to immunotherapy that use two or more monotherapies can significantly extend the utility of immunotherapies in the clinic (3, 6–9). Specific combinations of cytokine and co-inhibitory receptor agonists or antagonists have proven particularly efficacious in preclinical models of cancer and are now being tested in human trials (8, 10–15). However, this approach remains challenging because of the risks of exacerbated toxicity and the need for complex clinical trial design (6, 7). For cytokine-based therapies numerous challenges exist including pharmacokinetic barriers and immunogenicity, there is also the potential for the activation of inhibitory feedback pathways that can lead to immune suppression, all of which requires careful consideration (16–18).

Interleukin-21 is a type I cytokine and a member of the common cytokine receptor gamma-chain (cg-chain) family that has emerged as a promising immune therapeutic for the treatment of cancer (8). IL-21 that is produced by activated CD4+ T cells and natural killer T (NKT) cells signals via a heterodimeric receptor complex comprised of a discrete IL-21 receptor (IL-21R) subunit together with the cg-chain (19). Activation of the IL-21R complex leads to the activation of the JAK/STAT signaling pathway (20). IL-21R is broadly expressed in hematopoietic cells including T and B lymphocytes, natural killer (NK) cells and myeloid cells (20). Although not an essential growth or differentiation factor, IL-21 is a potent mitogen and survival factor for both NK cells and activated T cells (19, 20). IL-21 can support the differentiation of CD4 + T helper 17 (Th17) as well as follicular helper T cells (Tfh) and can antagonize regulatory T cell (Treg) differentiation. Additionally IL-21 augments the survival of CD8+ T cells resulting in a less activated but more persistent T cell phenotype that leads to enhanced tumor and viral control (8, 19–25). In B cells, IL-21 induces proliferation or apoptosis in a contextual manner and is involved in class switch recombination and optimal plasma cell differentiation (19, 20). A challenging facet of cytokine immunotherapy is that while activating immune cells to potentiate immune responses, the same cytokine can also activate counter-regulatory pathways as exemplified by IL-2 and IFNγ. These counter-regulatory pathways activate protective immune responses, regulatory T cell responses and inhibitory pathways such as PD-L1 (18, 26–32). In dendritic cells (DCs), IL-21 inhibits both maturation and activation and can induce the apoptosis of conventional DCs and in mixed cultures, can potently inhibit the priming of T cells, and may play a role in the induction of tolerance (17, 19, 20).

In humans, IL-21 has been tested as a non-targeted free cytokine in several cancer indications, but despite the promising preclinical data and early phase I clinical data, development of this approach has not progressed further than phase II testing (33, 34). More recently in preclinical models, combination of recombinant IL-21 cytokine together with co-inhibitory receptor antagonists, namely anti-CTLA4 and anti-PD-1 have demonstrated that IL-21 can extend the efficacy of these treatments, and these combinations are now being tested in the clinic (35). However, given the challenges of using cytokines as immunotherapies, it is possible that the preclinical efficacy observed with such combinations may not translate into the clinic. For the reasons discussed above, we hypothesized that to harness the immune potentiating activity of IL-21 it may be prerequisite to address the liabilities of this cytokine, including short half-life and off-target immune suppression. Toward this goal we devised a strategy focusing on an immunocytokine approach that would allow for the delivery of an engineered IL-21 cytokine, in a targeted manner that would circumvent potential liabilities, thus enabling improved exposures and maximizing efficacy.

MATERIALS AND METHODS

Protein Production

The recombinant fusion molecules were produced using a process similar to the process as described by Shi S. Y. et al. (36). Briefly, these molecules were cloned into a pTT5 expression vector and transiently transfected into HEK293-6e suspension cells. Conditioned medium was harvested 6 days post-transfection by centrifugation and then the molecules were purified from conditioned medium using MabSelect SuRe (GE Healthcare) and SP (GE Healthcare) cation exchange chromatography, before formulated into 10 mM acetic acid, 9% sucrose, pH 5.2.

Human, Cynomolgus Monkey and Mouse PD-1/IL21R Binding Affinity Characterization

IL21R and PD-1 binding affinity were quantitated with ForteBio Octet RED384 and Octet HTX instruments using 384 well plates at 27°C. Unless noted, Octet sample buffer was used for all sample dilution, baselines, association and dissociation steps (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.10 mg.ml BSA, 0.13% (v/v) Triton X-100).

IL21R Binding Affinity

Both monovalent IL21R-FLAG-His and bivalent IL21R-Fc recombinant reagents were tested but produced very similar results (within ∼2–3 fold). Human IL21R(1-232)-FLAG-His, cyno IL21R(1-232)-FLAG-His and mouse IL21R(1-236)-FLAG-His were minimally biotinylated (∼1–2 bn/mol) and captured on Streptavidin SAX biosensor tips to a 2.0 nm loading level. The biosensor tips were then incubated in wells containing the anti-PD-1 antibody x IL21 samples in a 3-fold serial dilution. For wildtype IL21 cytokine fusions, the top antibody fusion sample concentration was 10 nM, while for IL21 cytokine mutein fusions the top antibody fusion concentration was 300 nM. An

association time of 20 min and a dissociation time of 1.5 h was used to maximize curvature in the active binding sensorgrams for more accurate kinetic fits.

PD-1 Binding Affinity

The anti-PD-1 × IL21 antibody fusions were immobilized on amine reactive AR2G biosensor tips through EDC-NHS activation (600 s) followed by immobilization (15 – 20 nM proteins at pH 6 for 2000 s) and then quenched (1 M Ethanolamine, 300 s). After immobilization, the biosensor tips were incubated in Octet running buffer for 300 s (baseline). The final immobilization level for the anti-PD-1 × IL21 antibody fusions was at least 2 nm. The immobilized biosensor tips were then incubated in wells containing a 3-fold serial dilution of the soluble, recombinant PD-1 receptors for human PD-1 (1-170)-FLAG-His, cynomolgus PD-1 (1-167)-FLAG-His or mouse PD-1(25-167)-His (R&D Systems catalog #9047-PD). In all cases, the top PD-1 concentration was 30 nM. Association for 300 s and dissociation for 500 s were used since they empirically produced enough curvature for accurate kinetic fits.

All ForteBio Octet raw data was processed using the ForteBio Data Analysis software v9, v10, or v11: (a) two reference tip curves which had immobilized target but no interaction (i.e., Octet buffer only) were averaged and subtracted from the remaining sample tips curves in the same column; (b) the association and dissociation curves were isolated and aligned to the *Y* axis; (c) the association and dissociation interstep were aligned; (d) Savitzky-Golay filtering was implemented to reduce the high-frequency noise and (e) the resulting set of association and dissociation curves for each sample-target interaction were globally fit with a 1:1 binding model to determine the measured values of the association rate constant k_a (units M^{-1} sec^{-1}) and the dissociation rates constants k_d (unit sec^{-1}); the equilibrium dissociation constant K_D (units M) was calculated as a ration of the dissociation and association rates constants ($=k_d/k_a$).

Subcutaneous CT26 Tumor Model

Eight-week-old female BALB/c mice (Charles River Laboratories, Hollister, CA, United States) were injected subcutaneously on the right hind flank with 3×10^5 CT26 cancer cells (CRL-2639, ATCC) in 0.1 mL of RPMI media on study day 0. On day 12, tumor volumes were determined, mice were randomized into study groups of ten animals per group, and treatments were initiated: IgG1 isotype 300 μg intraperitoneal (IP) Q3Dx3, anti-PD-1 300 μg IP Q3Dx3, rmIL-21 50 μg IP 3x weekly for 3 weeks, or a combination of rmIL-21 and anti-PD-1. Tumor volumes were measured twice per week. All experimental studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Amgen (IACUC). Animals were housed at Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-accredited facilities (at Amgen) in ventilated micro-isolator housing on corncob bedding. Animals had access *ad libitum* to sterile pelleted food and reverse osmosis-purified water and were maintained on a 12:12 h light:dark cycle with access to environmental enrichment opportunities.

Humanized Mouse Model Reconstituted With Human CTLs

NOD.Cg-Prkdc^{scid} Il2rg$^{TM1\,Wjl}$/SzJ (Jax stock number 005557) were used at 6–8 weeks of age. On day 0, animals were reconstituted with 2.5×10^6 freshly thawed CTLs in 100 μl in PBS by retro-orbital injection, 2×10^5 EU IL-2 (Peprotech, catalog # 200-02-1 mg, lot# 11172) in 0.02% BSA in PBS in 100 μl by intraperitoneal injection, and 1×10^6 CMV peptide-expressing luciferase-labeled SKMEL-30 melanoma cells (CMV-SKMEL30-Luc) tumor cells in 100 μl in a 50:50 mixture of growth factor reduced Matrigel (Corning) and serum-free RPMI subcutaneously on the right hind flank. CMV-SKMEL30-luc cells were transduced with the CMV antigens pp65, IE1, and UL138 by lentiviral transduction and blasticidin resistance was used as a selection marker (lentivirus was generated by Applied Biological Materials). The cell line was then luciferase labeled using lentivirus and puromycin selection, MAP tested (IDEXX), and expanded for use *in vivo*. IL-2 was reconstituted according to manufacturer's protocol. Animals received two additional boosters of IL-2 on d2 and d11. On day 17, tumor volumes were determined, mice were randomized into study groups of ten animals per group, and treatments were initiated: Isotype 300 μg IP Q3Dx3 (BioXCell), anti-PD-1 mAb3 (chimera consisting of anti-human PD-1 variable region and mouse IgG1 constant region) 300 μg IP Q3Dx3, anti-PD-1 mAb3 x R9E:R76A (chimera consisting of anti-human PD-1 variable region, a mouse IgG1 constant region and a C-terminus fusion of human IL-21 variant R9E:R76A) fusion protein monomer 363 μg IP Q3DX3. Tumor volumes were measured twice/week. All experimental studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Amgen. Animals were housed at Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities (at Amgen) in ventilated microisolator housing on corncob bedding. Animals had access *ad libitum* to sterile pelleted food and reverse osmosis-purified water and were maintained on a 12:12 h light:dark cycle with access to environmental enrichment opportunities.

Cynomolgus Monkey Studies

Experimentally naïve cynomolgus monkeys, 2 to 5 years of age, and weighing 2.7 to 5.7 kg at the onset of the study, were assigned to dosing groups. Blood samples were drawn for pharmacokinetic analysis prior to the first dose and at 0.083, 0.25, 1, 24, 72, 120, 168, 240, and 336 h after a single dose. Serum was separated from blood samples and stored frozen at -80°C and the resulting cell pellet underwent red cell lysis. Serum samples were analyzed for intact drug and the following pharmacokinetic parameters were evaluated from the serum samples: the terminal half-life calculated from the terminal slope of the log concentration-time curve ($t_{1/2}$), maximum concentration (C_{max}), the time of peak plasma concentration (T_{max}), and area under the curve (AUC).

Cynomologus monkey studies were conducted under protocols approved by the Charles River Laboratories IACUC. Animals were housed at AAALAC-accredited facilities (Reno, Nevada).

In vitro STAT3 Phosphorylation

HuT78 (ATCC, TIB-161) and HuT78 PD-1 stable cell lines are serum starved for 16 h. HuT78 parental and HuT78 PD-1 stable cell lines (transduced with human PD-1) were then seeded onto separate plates at 40,000 cells per well in the presence of serially diluted antibodies in triplicate for 40 min at 37° C., 5% CO^2. pSTAT3 Tyr705 levels were measured using AlphaLISA Surefire Ultra pSTAT3 (Tyr705) Assay Kit (Perkin Elmer, #ALSU-PST3-A10K).

PD-1 Reporter Assay

GloResponse Jurkat NFAT-*luc2*/PD-1 stable effector cells (Promega, #CS187102) and the CHO PD-L1 stable cell line (Promega, #CS178103) were co-cultured at a ratio of 1.25:1 in the presence of serially diluted antibodies in triplicate for 6 h at 37° C., 5% CO^2. Luminescence was measured using Bio-Glo Luciferase Assay System (Promega, #G7940).

Mixed Lymphocyte Culture

Mismatched donor pair leukopaks were obtained from AllCells Inc., Donor's T cells were isolated using Pan T-cell Isolation Kit (Miltenyi Biotec, # 130-096-535) and a mismatched donor's monocytes were isolated using Pan Monocyte Isolation Kit (Miltenyi Biotec, #130-096-537). Monocytes were further matured for 10 days using CellXVivo Human Monocyte-Derived Dendritic Cell Differentiation Kit (R&D Systems, #CDK004). Pan-T cells were co-cultured with matured monocytes at a ratio of 10:1 in the presence of serially diluted antibodies in triplicate for 72 h at 37° C., 5% CO^2. Supernatant IL-2 levels were measured by ELISA (Mesoscale Discoveries, #K151QQD-4).

In vitro B Cell Stimulation

Frozen human peripheral blood mononuclear cells (PBMCs) from normal donors were obtained from AllCells, Inc. (Alameda, CA, United States). Frozen cynomolgus PBMCs were obtained from SNBL USA, Ltd. (Everett, WA, United States). To assess the phosphorylation of STAT3 in a mixed human or cynomolgus cell population in response to anti-PD-1-IL21 treatment, frozen human or cynomolgus PBMCs were gently thawed, washed and resuspended with HBSS buffer. Cells were plated onto 96-well round-bottom polypropylene plates at 3–5 × 10^5 cells/well and treated with various doses of anti-PD-1-IL21 or appropriate controls for 10 min at 37°C, 5% CO_2. Cells were then washed with cold staining buffer (PBS + 2% FBS) and labeled with Alexa Fluor 488-conjugated mouse αCD3 (SP34-2) (BD Biosciences #557705) followed by a fixable live-dead stain in accordance with the manufacturer's recommended protocol. Intracellular staining was achieved by fixing the cells with 200 μl of 1X Lyse/Fix Buffer (BD Bioscience #558049) per well for 10 min at 37°C, washing the cells twice with staining buffer, then permeabilizing with 200 μl of cold Perm III Buffer (BD Bioscience #558050) for 30 min on ice. After washing with staining buffer, the cells were stained with PE-conjugated mouse αStat3 (pY705) (BD Bioscience #612569). Cells were then washed twice with staining buffer and then analyzed by flow cytometry.

In vitro Cytotoxic T Cell Assay

Expansion of Cytomegalovirus (CMV) Antigen-Specific Cytotoxic T Lymphocytes (CTLs)

Cytomegalovirus antigen-specific CTLs were isolated from PBMCs of CMV seropositive donors. Monocytes were enriched (EasySep Human monocyte isolation kit, Stem Cell Technologies) from the donors and differentiated into dendritic cells (DCs) using the Human Dendritic Cell Differentiation Kit (R&D Systems). The DCs were then matured in the presence of TNF-alpha (R&D Systems), IL-6 (R&D Systems), IL-1 beta (Peprotech), Prostaglandin E2 (Acros organics) and 5 μg/ml pp65 CMV peptide (AnaSpec). Mature DCs were co-cultured with autologous PBMCs in G-Rex flasks (Wilson Wolf) at a ratio of 10:1 PBMC to DC in RPMI + 10% heat-inactivated FBS (Gibco) + 1X sodium pyruvate (Gibco) + 1X non-essential amino acids (Gibco) + 1X β-mercaptoethanol (Gibco). For some experiments cell were primed with 100 nM PD-1 mAb (Amgen) or 100 nM PD-1 X R9E:R76A monomer (Amgen) on day 2 post coculture or left untreated. To determine antigen specificity of the CTLs following expansion, cells were stained with iTAg Tetramer/PE– CMV pp65 tetramer (MBL) 5 days post priming and analyzed by flow cytometry.

FACS Characterization of CTLs

Seven days post co-culture, cells were collected, washed and counted. Single-cell suspensions were then stained with fluorochrome-conjugated antibodies and immunofluorescence was analyzed on a FACS Symphony (BD Biosciences) using standard techniques. Antibodies used in this experiment were: anti-CD3(clone:SK7, BD Biosciences) anti-CD8(clone:SK1, BD Biosciences); anti-CD28 (clone:CD28.2, BD Biosciences); anti-CD62L (clone: DREG56, Biolegend); anti-Ki67 (clone:B56, BD Biosciences); anti-CXCR5(clone:RF8B2, BD Biosciences) and anti-PD-1(Amgen).

CTL Killing Assay and IFN-Gamma Expression

Nine days post coculture, CD8(+) T cells were enriched from the PBMC: DC cultures and CMV specific CTLs were FACS sorted using standard protocol. Sorted cells were resuspended in RPMI + 5% heat-inactivated FBS (Gibco) + 1X sodium pyruvate (Gibco) + 1X non-essential amino acids (Gibco) + 1X β-mercaptoethanol (Gibco) and rested overnight. The cells were then added into 96-well black-wall clear-bottom plates (Corning) containing pp65 CMV peptide-pulsed luciferase-labeled SKMEL-30 melanoma cells at an effector to target ratio of 2:1. After a 36-h incubation, specific lysis was assessed by adding Bio-Glo reagent (Promega) and reading the plates on the BioTek Synergy Neo2 plate reader (BioTek instruments) using standard luminescence. The supernatants from the above cultures were collected, and IFN-gamma levels were assessed according to manufacturer's protocol (Meso Scale discovery). In brief, dilution series of controls (detection limit 20,000 pg/mL) and cell culture supernatant (25 μl per well) were transferred to pre-blocked (with 1%w/v solution of Blocker B in PBS) IFN-gamma capture antibody-coated plates and incubated for 2 h at RT, followed by addition of IFN-gamma detection antibody and further incubation of 2 h at RT. The plates were then washed thrice with

PBS-0.05% Tween and after addition of read buffer T, the plates were read using a MESO SECTOR S600 (Meso Scale Discovery).

Alternative CTL Killing Assay

1 × 10^6 CMV-specific CTLs were washed and resuspended in X-VIVO 15 media (Lonza) then plated in 24-well TC-treated plates (Corning) that have been coated with 0.5 μg/ml anti-CD3 (BioLegend) and 2.5 μg/ml anti-CD28 (BioLegend). Test molecules were added at a final concentration of 500 nM along with 10 U/ml IL-2 (R&D Systems). Following 7 days of incubation at 37°C/5% CO_2, CTLs were washed, resuspended in RPMI + 10% heat-inactivated FBS (Gibco) + 1X sodium pyruvate (Gibco) + 1X non-essential amino acids (Gibco) + 1X β-mercaptoethanol (Gibco), then titrated into 96-well black-wall clear-bottom plates (Corning) containing pp65 CMV peptide-pulsed luciferase-labeled SKMEL-30 melanoma cells beginning at an effector to target ratio of 20:1. After a 3 day incubation, specific lysis was assessed by adding Bio-Glo reagent (Promega) and reading the plates on the EnVision (PerkinElmer) using standard luminescence settings.

Statistical Analysis

Graphs were plotted, and statistical significance was established using GraphPad Prism version 7.04 (GraphPad Software, San Diego, CA, United States)[1]. For correlation analysis Pearson correlation co-efficient analysis was used. For comparison of survival curves log-rank (Mantel–Cox) test was used. A Non-linear curve fitting was done using variable slopes (four parameters) method on log-transformed data to establish half maximal effective concentration (EC50) values. Anova with a Tukey's multiple comparison test was used to calculate statistical differences between groups *in vitro* studies and to compare tumor volumes between the treatment groups. $p < 0.05$ (*) taken as statistical significance (**$p < 0.01$, ***$p < 0.001$, NS, non-significant).

RESULTS

Design of Anti-PD-1 and IL-21 Cytokine Fusion Proteins

Recombinant free IL-21 provides modest protection in various preclinical cancer models that is further amplified upon combination with other immune therapies (19, 35, 37). Using a subcutaneous mouse model of colon cancer, we confirmed a combination of recombinant free IL-21 and anti-PD-1 antibody (mAb) dosed concurrently, extended survival, in an established tumor model (**Supplementary Figure S1**A). IL-21R is expressed broadly throughout the hematopoietic system which significantly impacts cytokine biodistribution and the half-life. Cytokines can be engineered to improve pharmacokinetic properties and therapeutic index; however, most engineered cytokines have only modest improvements in pharmacokinetic properties and often still manifest dose-limiting toxicity and therefore remain constrained to dosing regimens below that of antibodies (15, 30, 38, 39). Moreover, in fusion proteins, the high affinity interaction between the cytokine and its cognate receptor can skew biodistribution away from the targeting antibody noted in previous studies (40). We assessed whether IL-21 could be targeted to PD-1-positive cells by generating antibody cytokine fusion proteins (anti-PD-1 mAb × IL-21) using an anti-PD-1 antibody and the unmodified IL-21 sequence. We avoided fusing the IL-21 cytokine to the N-terminus of the antibody heavy chain or the light chain since this could impact antibody binding to PD-1. We therefore decided to fuse IL-21 to the C-terminus of the antibody heavy chain to preserve bivalency and for optimal targeting. The lysine residue at the C-terminus of the antibody heavy chain was deleted to remediate any potential clipping (41). As depicted in **Supplementary Figure S2**A, we explored two different designs where the N-terminus of IL-21 was fused to the C-terminus of the antibody heavy chain either with or without (a glycine and serine) linker (GGGGS). In all cases the antibody Fc region was engineered to be devoid of interactions with FcgRs and C1q (SEFL2-2, **Supplementary Figure S2**A) (42). We confirmed that homodimer fusion proteins, both G4S-linker and linker free variants, could be expressed and we next proceeded to test the cell potency of the fusion molecules. For this we used an IL-21R expressing human T cell line (Hut78) or a variant of this cell line engineered to express PD-1 protein [Hut78 PD-1(+)]. Cells were stimulated with test articles and STAT3 transcription factor phosphorylation was monitored as a surrogate measure of IL-21 pathway activation. As expected, strong phosphorylation of STAT3 was observed in both Hut78 cell lines irrespective of PD-1 expression when they were stimulated with recombinant free WT IL-21 (**Supplementary Figure S2**B). For the fusion proteins, we observed mild but significant loss in potency and efficacy of STAT3 phosphorylation in the absence of PD-1 expression in the Hut78 parental cells (**Supplementary Figure S2**B). In contrast, in cells engineered to express cell surface PD-1, we observed complete restoration of STAT3 signaling with evidence for a mild improvement in potency as compared to WT free cytokine (**Supplementary Figure S2**B). From these results, we determined that fusion of IL-21 to the C-terminus of an antibody can serve to partially attenuate cytokine activity in manner that can be restored by antibody mediated targeting of cell surface PD-1 antigen (**Supplementary Figure S2**B) (43). Antibody cytokine fusion proteins are known to have altered pharmacokinetic (PK) properties as compared to monoclonal antibodies or recombinant free cytokines. To understand how fusion of IL-21 cytokine to a mAb domain can alter pharmacokinetic properties *in vivo,* we next examined PK properties of a fusion protein consisting of an anti-PD-1 mAb and WT IL-21 (anti-PD-1 × IL-21 WT, homodimer). Anti-PD-1 × IL-21 WT or mAb domain was dosed intravenously into cynomolgus monkeys (**Supplementary Figure S3A**). The results as shown in **Supplementary Figure S3**B, demonstrate that as compared to the parent anti-PD-1 mAb the fusion protein has significantly lower exposures and shortened half-life. We thus hypothesized the abundance of IL-21R positive cells, expressed broadly on hematopoietic cells, coupled with the high affinity of the cytokine domain for its cognate receptor is likely to

[1]http://www.graphpad.com

be the primary determinant of biodistribution properties of the fusion protein.

Design and Characterization of Single Amino Acid Substitution IL-21 Variants

To restrict cytokine activity to targeted cells and thereby further improve PK properties and therapeutic index, we decided to implement a strategy in which the affinity of the IL-21 cytokine for IL-21R was attenuated, our strategy is outlined in **Supplementary Figure S4**. It is expected that under these conditions cytokine activity can only be delivered in *cis* upon a stabilized interaction between cytokine and cognate receptor, which is enabled by binding of the antibody domain to the targeted cell surface protein. We next proceeded to generate a panel of IL-21 muteins fused to an anti-PD-1 mAb using the linker free homodimer format. Structure guided engineering was used to create a panel of 101 *muteins* each having a single amino acid substitution in the IL-21 amino acid sequence (**Table 1**). We focused on key amino acid residues in IL-21 that are conserved across (human and cynomolgus monkey) species and mediate the interaction between IL-21 and IL-21R. For the identification of residues that could be mutated to attenuate IL-21 binding to the IL-21R, we utilized the published co-crystal structure of the IL-21: IL-21R complex (PDB ID: 3TGX) (44). Residues within the IL-21: IL-21R were identified and selected for *in silico* mutagenesis to generate a panel of muteins in which each of the selected residues was changed to one of sixteen alternate amino acid residues (except cysteine, phenylalanine and tryptophan), using MODELER tool (Biovia Discovery Studio) to optimize conformation. In order to quantify the probable impact of each mutation on the binding of IL-21 to IL-21R, $\Delta\Delta G_{mut}$ (where $\Delta\Delta G_{mut}$ is the difference between the calculated binding free energy, $\Delta\Delta G_{bind}$, of the mutated structure and the wild type structure and $\Delta\Delta G_{bind}$ is the difference between the free energy of the complex and the unbound proteins) was calculated by using the Biovia Discovery Studio software (45). Mutations that led to $\Delta\Delta G_{mut} > 1$ kcal/mol were selected for further analysis. Further residues for mutation were also identified by visual inspection of the IL-21: IL-21R complex structure (PDB ID: 3TGX) and the unbound structure (PDB ID: 2OQP) of IL-21 (44). Additional residues were selected in region 56-83 (residues R65, I66, V69, S70, K72, K73, K75, R76, K77, and S80) of IL-21 which has previously been reported to exhibit partial helix and disorder forms, and is present in the IL-21R binding interface (46). Each of the selected residues within region 56–83 were mutated to glycine and proline residues with the goal of disrupting the helix structure of this region to disfavor the bound conformation of the IL-21 (**Table 1**). Biophysical and functional properties of the fusion proteins were determined, and for the IL-21 domain these attributes were compared to those of the WT free cytokine (**Table 2** and **Figure 1**). Equilibrium dissociation constant (K_D) was determined for IL-21R for free WT IL-21 and for each of the fusion proteins (**Table 2**). Since mutations in the IL-21 receptor binding domain impinge on the affinity of the cytokine for IL-21R, it was not possible to assign an accurate K_D in many of the muteins (**Table 2**).

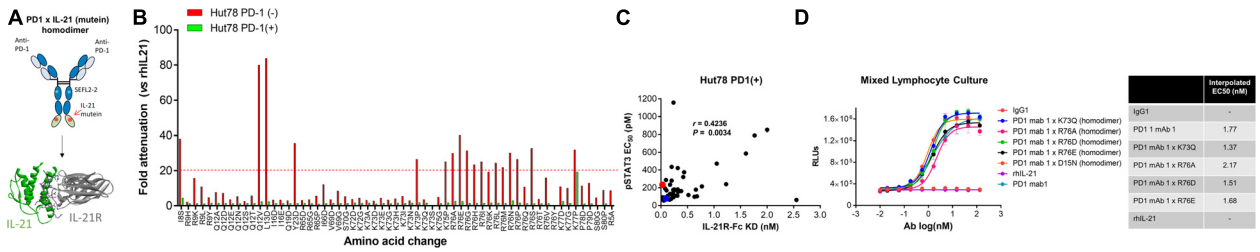
TABLE 1 | IL-21 residues selected for substitution.

	IL-21 aa																			
Position	**I16**	**I66**	**I8**	**K72**	**K73**	**K75**	**K77**	**L13**	**P78**	**Q12**	**Q19**	**R5**	**R65**	**R76**	**R9**	**S70**	**S80**	**V69**	**Y23**
Substituion	I16D	I66D	I8A	K72D	K73A	K75D	K77D	L13D	P78D	Q12A	Q19D	R5A	R65D	R76A	R9A	S70E	S80G	V69D	Y23D
	I16E	I66G	I8D	K72G	K73D	K75G	K77G		P79D	Q12D		R5D	R65G	R76D	R9D	S70G	S80P	V69G	
		I66P	I8E	K72P	K73E	K75P	K77P			Q12E		R5E	R65P	R76E	R9E	S70P		V69P	
			I8G		K73G					Q12N		R5G		R76G	R9G	S70Y			
			I8N		K73H					Q12S		R5H		R76H	R9H				
			I8S		K73I					Q12T		R5I		R76I	R9I				
					K73N					Q12V		R5K		R76K	R9K				
					K73P							R5L		R76L	R9L				
					K73Q							R5M		R76M	R9M				
					K73S							R5N		R76N	R9N				
					K73V							R5Q		R76P	R9Q				
												R5S		R76Q	R9S				
												R5T		R76S	R9T				
												R5V		R76T	R9V				
												R5Y		R76V	R9Y				
														R76Y					

TABLE 2 | IL-21 *mutein* binding to human IL-21R.

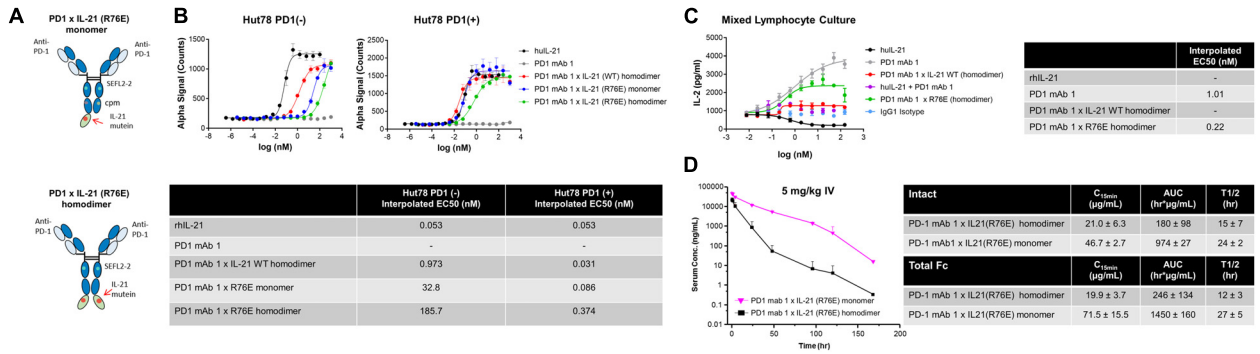
	IL21R-Fc		IL21R-Fc
Variant	**KD (nM)**	**Variant**	**KD (nM)**
rhIL-21	0.027	V69D	0.040
Anti-PD-1 mab 1 x IL-21 WT	0.079	V69G	0.21
R5D	No binding	V69P	2.0
R5E	No binding	S70E	0.95
R5G	No binding	S70G	0.52
R5G	Weak binding	S70P	∼10
R5I	Weak binding	K72D	0.24
R5K	Weak binding	K72G	0.25
R5L	Weak binding	K72P	9.0
R5M	Weak binding	K73A	0.053
R5N	No binding	K73D	0.44
R5Q	2.100	K73E	0.073
R5S	Weak binding	K73G	0.25
R5T	Weak binding	K73H	0.19
R5V	Weak binding	K73I	0.17
R5Y	Weak binding	K73N	0.074
I8A	Weak binding	K73P	∼2
I8D	Weak binding	K73Q	0.069
I8E	No binding	K73S	0.17
I8G	weak binding	K73V	1.1
I8N	Weak binding	K75D	20
I8S	−4	K75G	0.16
R9A	6.836	K75P	∼1
R9D	>100	R76A	∼11
R9E	No binding	R76D	∼12
R9G	−40	R76E	18
R9H	0.084	R76G	∼2
R9I	2.2	R76H	∼2
R9K	2.0	R76I	0.32
R9L	1.6	R76K	∼0.2
R9M	Weak binding	R76L	∼0.2
R9N	Weak binding	R76M	∼0.6
R9Q	Weak binding	R76N	15
R9S	Weak binding	R76P	∼0.4
R9T	Weak binding	R76Q	0.77
R9V	Weak binding	R76S	1.1
R9Y	0.063	R76T	0.11
Q12A	0.23	R76V	1.8
Q12D	0.42	R76Y	0.27
Q12E	0.031	K77G	0.66
Q12N	0.38	K77P	2.1
Q12S	0.32	P78D	1.2
Q12T	<0.26	P79D	0.32
Q12V	<2.2	S80G	0.27
L13D	11	S80P	0.31
I16D	0.094	R5A	0.24
I16E	0.076	S70Y	0.24
Q19D	0.17		
Y23D	1.7		
R65D	0.088		
R65G	0.13		
R65P	0.90		
I66D	0.68		
I66G	2.6		
I66P	7.1		

We also tested the *in vitro* activity of the fusion molecules using our engineered Hut78 cell lines. We report that in agreement with the binding data, because of the high degree of attenuation for IL-21R, we observed attenuated STAT3 phosphorylation in the absence of PD-1 expression in the Hut78 parental cells. In contrast, in cells engineered to express cell surface PD-1, we observed significant restoration of STAT3 signaling, but signaling was still partially attenuated as compared to free wildtype cytokine (**Figure 1B**). For those muteins where we could measure both affinity and potency in Hut78 PD-1 expressing cells, we were able to confirm a positive correlation between cell activity and the affinity of the molecules (**Figure 1C**). To test the blocking activity of the PD-1 mAb arm of the fusion protein, we used a reporter gene assay (Promega) in which PD-1-expressing Jurkat effector cells are incubated with antigen presenting cells expressing PD-L1 in the absence or presence of PD-1 blocking antibodies. The results, shown for a subset of the fusion proteins, suggest that the fusion proteins retain the ability to bind and block the PD-1 pathway with similar potency to the parent anti-PD-1 mAb (**Figure 1D**). For a more detailed characterization of the impact of mutations that disrupt binding of IL-21 to IL-21R, we selected a single anti-PD-1 × IL-21 variant (R76E) which had preferred attributes of high attenuation of activity in PD-1 (−) but retained significant activity in PD-1 expressing cells. In addition, since improving pharmacokinetic properties is important for both sustained blockade of the PD-1 pathway as well providing a more prolonged IL-21 signal, and since it has previously been demonstrated that the valency of Fc-fusion proteins can significantly affect PK properties, we decided to test differences between a homodimer versus a monomeric IL-21 fusion proteins (47). Variant anti-PD-1 x R76E was cloned and expressed with IL-21 domain fused to each heavy chain resulting in a fusion molecule with two IL-21 domains (homodimer), and a configuration where the IL-21 domain was fused to only one of the heavy chains resulting in a fusion molecule with only one IL-21 domain (monomer). In the case of IL-21 monomer, to achieve a heterodimer consisting of a single IL-21 subunit and a bivalent mAb, charge pair mutations (cpm) in the Fc domain were used to drive heterodimeric association of the individual heavy chains of the mAb domain (**Figure 2A**) (43). As shown in **Figure 2B**, variant anti-PD-1 × R76E has attenuated IL-21 activity on PD-1 (−) cells which is restored upon PD-1 expression. Compared to a WT IL-21 fusion protein, the anti-PD-1 × R76E (monomer and homodimer) has a more attenuated activity. In addition, we find that the monomer variant of anti-PD-1 × R76E has a modest improvement in potency over the homodimer variant. We next wanted to understand using a more complex *in vitro* system the potential for off-target activation of non-targeted IL-21R expressing cells, as it is known that IL-21 can be immunosuppressive when exposed to antigen presenting cells, and can potently inhibit the alloresponse in mixed lymphocyte cultures (MLC, **Figure 2C**) (17). As shown in **Figure 2C**, we confirm that free IL-21 can potently suppress the alloresponse and that IL-21-mediated immune suppression is dominant when free-IL-21 and anti-PD-1 are combined as monotherapies. We also tested WT and anti-PD-1 × R76E variants of IL-21 fusion proteins, and report that



	Interpolated EC50 (nM)
IgG1	-
PD1 1 mAb 1	1.77
PD1 mAb 1 x K73Q	1.37
PD1 mAb 1 x R76A	2.17
PD1 mAb 1 x R76D	1.51
PD1 mAb 1 x R76E	1.68
rhIL-21	-

FIGURE 1 | Anti-PD-1 × IL-21 fusion proteins with attenuated IL-21 activity. **(A)** Schematic representation of fusion protein domain assembly. **(B)** IL-21 activity of fusion proteins (tested at 3.7 nM) monitored by STAT3 phosphorylation using AlphaLISA in variants of Hut78 T cells either parental PD-1 (–) or engineering PD-1 (+) cells. Activity of muteins expressed relative to WT recombinant human IL-21 as a measure of attenuation. **(C)** Correlation between potency monitored using STAT3 phosphorylation in PD-1 (+) Hut78 T cells (monitored by AlphaLISA) and binding (determined by Octet) to human IL-21R. *P*-value for ***c*** Pearson correlation co-efficient, $P = 0.0034$ and $r = 0.4236$. **(D)** Potency of single amino acid substitution IL-21 variants fused to anti-PD-1 mAb as homodimer and parental mAb in blocking PD-1/L1 interaction monitored using Promega PD-1/L1 bioassay.



	Interpolated EC50 (nM)
rhIL-21	-
PD1 mAb 1	1.01
PD1 mAb 1 x IL-21 WT homodimer	-
PD1 mAb 1 x R76E homodimer	0.22

	Hut78 PD1 (-) Interpolated EC50 (nM)	Hut78 PD1 (+) Interpolated EC50 (nM)
rhIL-21	0.053	0.053
PD1 mAb 1	-	-
PD1 mAb 1 x IL-21 WT homodimer	0.973	0.031
PD1 mAb 1 x R76E monomer	32.8	0.086
PD1 mAb 1 x R76E homodimer	185.7	0.374

Intact	C_{15min} (µg/mL)	AUC (hr*µg/mL)	T1/2 (hr)
PD-1 mAb 1 x IL21(R76E) homodimer	21.0 ± 6.3	180 ± 98	15 ± 7
PD-1 mAb1 x IL21(R76E) monomer	46.7 ± 2.7	974 ± 27	24 ± 2
Total Fc	C_{15min} (µg/mL)	AUC (hr*µg/mL)	T1/2 (hr)
PD-1 mAb 1 x IL21(R76E) homodimer	19.9 ± 3.7	246 ± 134	12 ± 3
PD-1 mAb 1 x IL21(R76E) monomer	71.5 ± 15.5	1450 ± 160	27 ± 5

FIGURE 2 | Characterization of IL-21 R76E single substitution variant with attenuated activity. **(A)** Schematic representation of monomer IL-21 (upper panel) and homodimer (lower panel) fusion proteins. **(B)** IL-21 activity of R76E variant or free WT IL-21, monitored using STAT3 phosphorylation (AlphaLISA) in Hut78 PD-1(–) cells (left panel) and engineered Hut78 PD-1 (+) cells (right panel). **(C)** Activity of free WT IL-21, fusion proteins or parental mAb in mixed lymphocyte cultures of alloreactive pan-T cells and dendritic cells. *N* = 2 cynomolgus monkeys/group in **c** dosed 5 mg/kg with single dose of homodimer or monomer IL-21 fusion proteins. **(D)** Mean plasma concentration-time profiles of anti-PD-1 × R76E monomer and anti-PD-1 × R76E homodimer fusion proteins (upper panels), with summary of pharmacokinetic parameters (lower panels).

TABLE 3 | IL-21 residues selected for substitution and generation of double mutants.

Double mutants
R9E, R76E
R9A, R76E
R5E, R76E
R5A, R76E
R5Q, R76E
R9E, R76A
R9A, R76A
R5E, R76A
R5A, R76A
R5Q, R76A

in the absence of any attenuation WT IL-21 fusion protein can also potently suppress the alloresponse response. When we examined the behavior of anti-PD-1 × R76E variant, we observe a subtle but significant suppression of the response at higher concentrations. To determine the *in vivo* characteristics of the anti-PD-1 × R76E variants, pharmacokinetic parameters (PK) were determined using cynomolgus monkeys. As shown in **Figure 2D, both monomer** and **homodimer** variants exhibit distinct PK profiles, with the monomer showing superior exposures and half-life. To address potential liabilities relating to non-specific signaling that could translate into immune suppression mediated through the action on dendritic cells, and to further explore the potential for using a monomeric format to improve pharmacokinetic properties, we proceeded to generate more attenuated IL-21 variants.

Design and Characterization of Dual Amino Acid Substitution IL-21 Variants With Reduced Off-Target Signaling

To further reduce non-specific IL-21 signaling, a second panel of molecules was constructed (**Table 3**). Using the known IL-21/IL-21R structure to help guide selection, single amino acid substitution variants with the greatest degree of attenuation, as determined using cell and binding assays were combined to create a panel of double mutant variants fused as a monomer or

TABLE 4 | Summary of *in vitro* attributes of anti-PD-1 x IL-21 double *muteins*.

	Hut78 PD-1 (–) Interpolated EC50 (nm)	Hut78 PD-1(+) Interpolated EC50 (nm)	PD-1 reporter Interpolated EC50 (nm)	Mixed lymphocyte culture Interpolated EC50 (nm)	huIL-21R KD (nM)*	cyIL-21R KD (nM)*	huPD-1 KD (nM)*	cyPD-1 KD (nM)*
IgGl	–	–	–	–	–	–	–	–
rhIL-21	0.003	0.002	–	–	0.029	0.044	–	–
PD-1 mAb 2	–	–	0.487	0.161	–	–	0.90	1.33
PD-1 mAb 2 x R5Q:R76E homodimer	>1000	1.1	0.367	0.518	>300	>300	0.68	1.27
PD-1 mAb 2 x R5Q:R76E monomer	>1000	0.28	0.809	0.249	>300	>300	0.56	1.28
PD-1 mAb 2 x R9E:R76A homodimer	>1000	4.42	0.308	0.625	>300	>300	0.71	1.42
PD-1 mAb 2 x R9E:R76A monomer	>1000	0.78	0.503	0.241	>300	>300	0.90	1.61

Sensograms are shown in* ***Supplementary Figure S7**.*

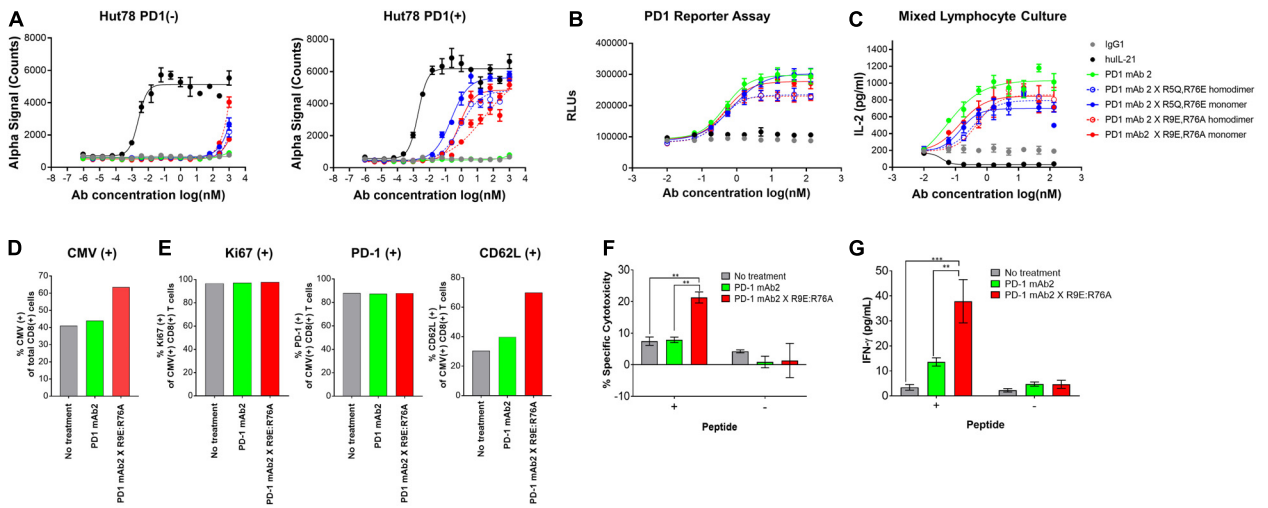


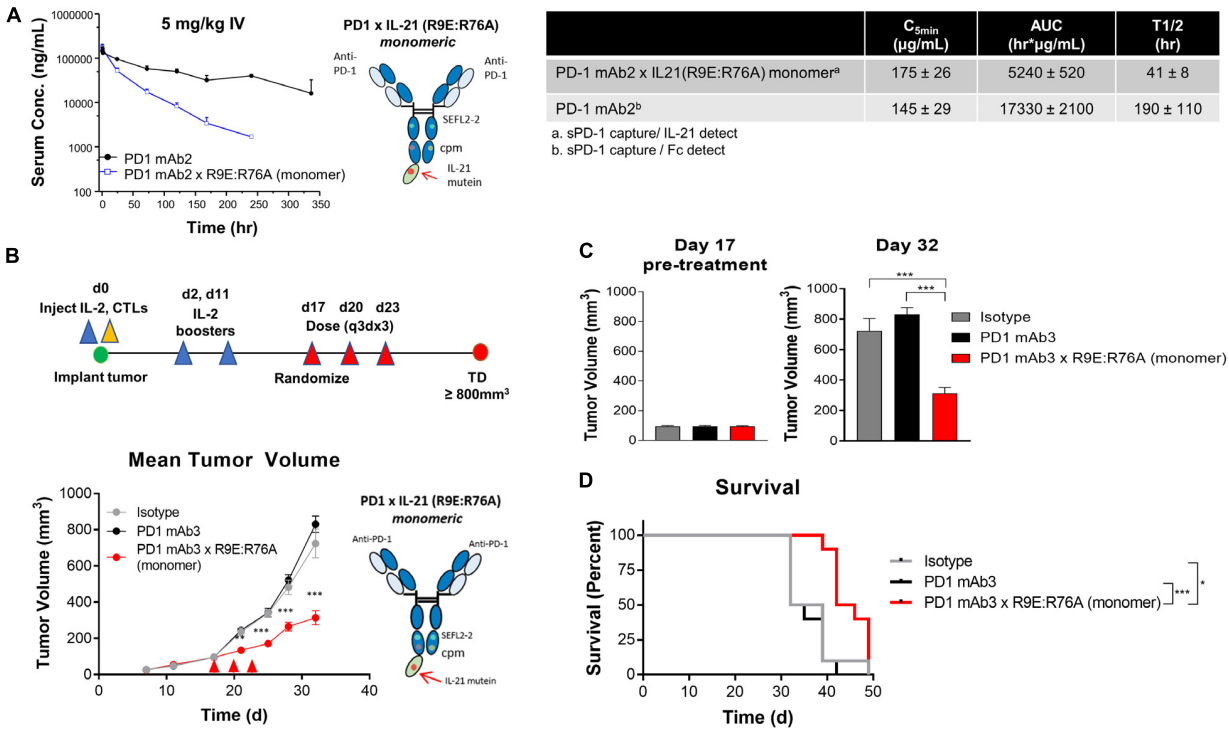
FIGURE 3 | Characterization of IL-21 variants with dual amino acid substitutions. **(A)** Activity of fusion proteins and free WT IL-21 in Hut78 PD-1(–) cells (left panel) and engineered Hut78 PD-1 (+) cells (right panel) monitored using STAT3 phosphorylation (AlphaLISA) as a surrogate measure of IL-21 activity. **(B)** Potency of fusion proteins and parental mAb in blocking PD-1/L1 interaction monitored using Promega PD-1/L1 bioassay. **(C)** Activity of free WT IL-21, dual amino acid substation variants or parental mAb in mixed lymphocyte cultures of alloreactive pan-T cells and dendritic cells. **(D–E)** CTLs derived from PBMCs from Donor 1 under indicated priming conditions. Seven days post co-culture, proportions of **(D)** CMV antigen specific CTLs and **(E)** Ki67 (left-panel), PD-1 (middle-panel) and L-selectin (CD62L, right-panel) was analyzed by FACS analysis. CTLs primed under different conditions were isolated and cocultured with peptide pulsed melanoma cells for 36 h to examine **(F)** cytotoxicity against peptide-loaded melanoma cells, determined by measuring luciferase activity and **(G)** CTL IFN-gamma production. Experiments in **(F)** and **(G)** were conducted in triplicates and the error bars represent SEM. *P*-values were calculated using one-way Anova with a Tukey's multiple comparison test. *P*-values: *** < 0.001, ** < 0.01.

homodimer to the C-terminus heavy chain of a bivalent anti-PD-1 antibody (**Table 3** and **Supplementary Figure S5**A). A subset of the double mutant variants was evaluated for binding to IL-21R (**Table 4** and **Supplementary Figure S7**). Consistent with the greater degree of attenuation, we were unable to establish K_D values for the interaction between fusion protein(s) and IL-21R, and we determined that these values are higher than the top concentration in the assay (300 nM), as such relative attenuation as compared with free WT cytokine is estimated to be >1000 fold for these more attenuated molecules (**Table 4** and **Supplementary Figure S7**). We next tested cell activity assays using a smaller subset of the double mutant constructs (**Figure 3** and **Supplementary Figures S5b,c**). According to our hypothesis, cell association of fusion proteins in which IL-21/IL-21R association has been disrupted can be restored through binding of the mAb domain to a cell surface receptor allowing for the stabilized interaction between IL-21 and IL-21R. In line with our hypothesis, double muteins demonstrate a high degree

of attenuation (>1000 fold as compared with free WT IL-21 cytokine) for STAT3 activation in cells devoid of PD-1 expression (**Figure 3A** and **Supplementary Figure S6**). Activity can be restored in cells engineered to express cell surface PD-1, but still partially attenuated as compared with free wildtype cytokine (**Figure 3A** and **Table 4**). We also confirmed that the fusion proteins consisting of the more attenuated IL-21 variants retain the ability to block the PD-1/PD-L1 interaction (**Figure 3B** and **Table 4**). We next proceeded to test if additional attenuation could protect against non-specific activation of bystander IL-21R expressing APCs in a mixed culture system using alloreactive T cells that respond to antigen peptide complexes presented by dendritic cells (**Figure 3C** and **Table 4**). In contrast to recombinant WT IL-21 cytokine, which completely suppressed the alloresponse, the fusion proteins have similar activity to the parental anti-PD-1 mAb (**Figure 3C**). Our data suggest that in the absence of PD-1 expression the more attenuated fusion proteins fail to activate bystander cells expressing IL-21R in these conditions, and in the context of an alloresponse the fusion proteins have only limited signaling in *trans* allowing for the preservation of DC function. We next tested, the impact of PD-1 × IL-21 fusion proteins on the differentiation and effector function of cytotoxic T cells (CTL) derived from PBMCs. For this we tested the activity of PD-1 × IL-21 fusion protein using two CMV seropositive donors across four independent experiments (**Figures 3D–G** and a second independent donor **Supplementary Figure S8**). Antigen specific CTLs were generated by co-culturing of peripheral blood mononuclear cells (PBMCs) and autologous peptide-loaded DCs in the presence of a PD-1 × IL-21 fusion protein and for comparison, PD-1 mAb or untreated cell were used as controls. After 7 days of co-culture with DCs, cell surface and intracellular markers of T cell proliferation and activation were monitored on antigen-specific CD8+ T cells. We report that T cell priming in the presence of PD-1 × IL-21 fusion protein gives rise to a mild but reproducible increase in the frequency of antigen-specific CD8+ T cells as compared to untreated control or PD-1 mAb treatment groups (**Figure 3D** and **Supplementary Figure S8**A). The increase in the frequency of antigen-specific cells was not correlated to increase in proliferation as the percentage of antigen-specific CD8+ T cells expressing Ki67 was equivalent across all treatment groups. We examined two further cell surface markers, namely PD-1 and L-selectin (CD62L), as markers of T cell activation and differentiation, respectively. Similar proportions of PD-1 positive T cells were observed across all treatment groups and was consistent across independent donors (**Figure 3E** and **Supplementary Figure S8**A). We also monitored L-selectin a marker enriched on naïve and memory T cells. Previously it has been reported that IL-21 promotes the acquisition of alternative effector phenotype with increased L-selectin (48). We report that priming of T cells in the presence of PD-1 x IL-21 fusion protein leads to an increase in proportion of L-selectin (CD62L) positive CTLs as compared to PD-1 mAb and untreated treatment groups (**Figure 3E** and **Supplementary Figure S8**A). These data suggest that in the presence of PD-1 × IL-21 CTLs can acquire an effector phenotype but retain the naïve marker L-selectin. We next examined effector function of differentiated CTLs by co-culturing CTLs together with peptide-loaded tumor cells. We report that CTLs conditioned with PD-1 × IL-21 fusion protein demonstrated superior cytotoxicity and IFN-gamma production as compared to untreated control or those primed together with a PD-1 mAb (**Figures 3F–G** and **Supplementary Figure S8**). We extended our studies to examine the effect of PD-1 × IL-21 fusion proteins on cytotoxicity of differentiated effector cells (**Supplementary Figure S9**). For these studies, to more faithfully mimic the clinical setting, in which fusion protein is expected to augment pre-existing immune responses, we used *in vitro* differentiated mature CTL lines. These were activated with a combination of CD3/28 beads (to mimic a chronic activation conditions) together with either anti-PD-1 mAb or fusion protein after which the CTLs were co-incubated with peptide pulsed PD-L1 (+) cancer cells. Our data suggest that under these conditions, CTLs treated with fusion protein have superior effector functions including cytotoxicity and IFN-gamma production versus anti-PD-1 mAb (**Figures 3D–F** and **Supplementary Figures S8, S9**).

In vivo Characterization of Dual Amino Acid Substitution IL-21 Variants With Improved Pharmacokinetic Properties and Superior Efficacy *in vivo* in an Anti-PD-1 Refractory Setting

We next wanted to extend our observations to understand pharmacokinetic properties of the more attenuated dual amino-acid substitution IL-21 fusion proteins. For this we used monomer fusions proteins because of their superior PK properties; groups of animals were dosed with fusion protein or parental mAb and PK parameters were calculated. The results as shown in **Figure 4A** suggest that attenuated cytokine variants have substantially improved PK properties as compared to first generation anti-PD-1 × R76E *mutein* (**Figure 2**). We extended our observations to explore *in vivo* activity of our fusion proteins. Since human IL-21 does not cross-react with mouse IL-21R and in the absence of an appropriate mouse surrogate molecule, we decided to implement a humanized mouse system; for this we used humanized mice, which were engrafted with human (PD-L1+) melanoma cells (SKMEL-30-Luc) engineered to express a model antigen (CMV-SKMEL-30-Luc, expressing peptide antigen derived from cytomegalovirus, CMV) and either a human-mouse chimeric PD-1 mAb, with a variable domain recognizing human PD-1 and a constant Fc-region from mouse IgG1, or a fusion protein consisting of the same parent PD-1 mAb and a monomeric variant of human IL-21 R9E:R76A (**Figure 4B** and **Supplementary Table S1**). On the same day as tumor engraftment, mice received adoptively transferred antigen (CMV)-specific CTLs, which we confirmed, demonstrate potent *in vitro* cytotoxicity against the antigen-expressing cancer cells (**Figure 3F**). In this model, the failure of tumor reactive CTLs to control cancer growth leads to development of progressive tumors which are palpable by day 17. Therapeutic administration (into mice with ∼100 mm^3 established tumors) with an isotype control antibody or an anti-PD-1 mAb failed to resolve the disease or have any discernable impact on tumor growth, establishing this tumor model as both



	C_{5min} (μg/mL)	AUC (hr*μg/mL)	T1/2 (hr)
PD-1 mAb2 x IL21(R9E:R76A) monomer[a]	175 ± 26	5240 ± 520	41 ± 8
PD-1 mAb2[b]	145 ± 29	17330 ± 2100	190 ± 110

a. sPD-1 capture/ IL-21 detect
b. sPD-1 capture / Fc detect

FIGURE 4 | *In vivo* characterization of dual amino-acid substation IL-21 fusion proteins **(A)** Mean plasma concentration-time profiles (upper left panel) of variant anti-PD-1 × R9E:R76A monomer (depicted, upper right and parent anti PD-1 mAb, with summary of pharmacokinetic parameters (lower panels). **(B–D)** *In vivo* activity of fusion protein administered to humanized NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mouse model engrafted with human melanoma cell line CMV-SKMEL-30-Luc expressing cytomegalovirus (CMV) antigen and reconstituted with human CMV-reactive CTLs adoptively transferred by retro-orbital injection on day 0, followed by randomization of mice with established (100 mm^3 tumors) on day 17 (N = 10 mice per group) and therapeutic administration of either isotype control mAb, PD-1 mAb3 or PD-1mAb3 × R9E:R76A monomer fusion protein administered by intraperitoneal injections **(B)** Summary of experimental design (upper-panel) and *in vivo* activity as measured by tumor volume (lower-panel). P-values were calculated with one-way Anova with Tukey's *post hoc* test and were as follow; Day 21: P = 0.0023 (PD-1 mAb3 vs. PD-1 mAb3 × R9E:R76A monomer) and P = 0.0056 (Isotype vs. PD-1 mAb3 × R9E:R76A monomer); Day 24: P = 0.0001 (PD-1 mAb3 vs. PD-1 mAb3 × R9E:R76A monomer) and P = 0.0001 (Isotype vs. PD-1 mAb3 × R9E:R76A monomer); Day 28: P = 0.0001 (PD-1 mAb3 vs. PD-1 mAb3 × R9E:R76A monomer) and P = 0.0012 (Isotype vs. PD-1 mAb3 × R9E:R76A monomer); Day 32: P = 0.0001 (PD-1 mAb3 vs. PD-1 mAb3 × R9E:R76A monomer) and P = 0.0001 (Isotype vs. PD-1 mAb3 × R9E:R76A monomer). Repeated Measures two-way Anova with Tukey's *post hoc* test for entire curve: Isotype vs. PD-1 mAb3: NS. Isotype vs. PD-1 mAb3 × R9E:R76A monomer: P = 0.0001. PD-1 mAb3 vs PD-1 mAb3 × R9E:R76A: P = 0.0001. **(C)** Summary of tumor volume at randomization (day 17) and pre-treatment (left panel) and at day 32 (right panel) P-values were calculated using one-way Anova with a Tukey's *post hoc* test. P = 0.0001 (PD-1 mAb3 vs. PD-1 mAb3 × R9E:R76A monomer) and P = 0.0001 (Isotype vs. PD-1 mAb3 × R9E:R76A monomer). **(D)** Survival analysis of tumor bearing mice. P-values of log-rank (Mantel-Cox) test were as follow; P = 0.0037 (Isotype vs. PD-1 mAb 3 × R9E:R76A monomer), P = 0.0001 (PD-1 mAb 3 monotherapy vs. PD-1 mAb 3 × R9E:R76A monomer). P-values: *< 0.05, ***< 0.001.

"high bar" and PD-1 refractory (**Figures 4B,C**). In contrast, therapeutic administration of a PD-1 × IL-21 fusion protein (**Supplementary Table S1**), has a significant inhibitory effect on the tumor growth and improves overall survival (**Figures 4B–D**). Collectively our data support the idea that chronic activation of T cells can lead to a diminished anti-tumor immune response, and that administration of a fusion protein consisting of a PD-1-targeted IL-21 moiety can significantly extend the function of CTLs and support superior tumor control in a mouse model that is refractory to PD-1 mAb monotherapy.

DISCUSSION

Inhibitors of T cell coinhibitory receptors such as anti-PD-1 and anti-CTLA4, can improve antitumor immunity. However, most patients remain refractory to these therapies (6). The effectiveness of coinhibitory receptor antagonists maybe extended in combination with additional modalities, including cytokines that function through complementary mechanisms (6, 7, 15, 29). Cytokines are small proteins that are essential in shaping protective antitumor immune responses, however, the utility of cytokines in the clinic for cancer immunotherapy is limited, with only TNFα, IFNα, and recombinant IL-2 approved for a small number of cancer indications (29, 49, 50). The inclusion of cytokines into therapeutic regimens faces considerable challenges, largely due to dose limiting toxicities and short serum half-life (6, 29). Engineered fusion proteins, where cytokines are genetically fused to an IgG antibody or a fragment thereof, commonly known as immunocytokines, can significantly extend half-life of cytokines, and improve safety by enabling targeted delivery to a specific cell or tissue. In the case of cytokines that present systemic toxicity or those that can both potentiate as well as suppress immune responses, such as IL-2 and IL-21, an

immunocytokine approach can serve to harness the potentially beneficial biology whilst limiting any detrimental impact to the host (10, 26, 29, 31, 38, 49). Many possible configurations can be considered when designing immunocytokines that can specify the nature of how the cytokine interacts with its target cell population or the local environment (49). Depending on the desired outcome, cytokines can be enriched in the tumor environment through tumor cell targeting antigens, with the view to (in *trans*) activate infiltrating local immune cells. Alternatively, cytokines can be delivered in *cis* directly to immune cells that are known to be enriched in the tumor environment *via* cell surface receptors expressed on leukocytes (**Supplementary Figure S4**). Additional considerations when designing cytokine fusion proteins include the nature of the Fc receptor interaction and how the cytokine domain is fused through N or C terminus fusion to IgG heavy or light chain can also significantly influence outcomes such as target cell expansion versus depletion as well as biodistribution and efficacy. The technical challenges of implementing immunocytokines is exemplified by recent examples of IL-2 fusion proteins (40, 43). Fusion of an antibody and cytokine can have undesirable outcomes for both arms of the molecule. High-affinity association of cytokine and its cognate receptor can alter distribution in favor of fast clearance, and in cases where there is a large population of non-targeted, cytokine receptor-expressing immune cells, can lead to increased toxicity (40). In the case of a fusion protein where the antibody domain has a function in addition to acting as a targeting moiety, as in the case of an antagonist antibody such as anti-PD-1, where prolonged blockade of the targeted receptor is needed, this can lead to loss of target coverage and efficacy. Whilst cytokine attenuation can improve toxicity profiles, including acute toxicities, such as cytokine storm, chronic low-level activation of cytokine pathways can still lead to the same undesirable outcome in the longer term. Moreover, to achieve or maintain desirable dosing properties the nature of the attenuating mutations need to be carefully considered, as simply attenuating cytokine activity to remediate undesirable properties such as off-target interactions may not improve, and can even worsen PK properties of the molecule (26, 28, 29).

The IL-21 cytokine has generated considerable interest as a potential immunotherapy, but in addition to the liabilities common to all cytokines including a short-half life, IL-21 can also suppress dendritic cell function and by extension priming of immune responses (8, 19, 26, 33–35, 51, 52). Using a structure guided protein engineering approach, we have combined an engineered IL-21 cytokine domain and a PD-1 blocking antibody into a bifunctional fusion protein. To overcome the limitations of IL-21 cytokine and to improve efficacy, we have used an iterative approach to design IL-21 variants with increasing degrees of attenuation that are masked from binding to IL-21R in the absence of PD-1 receptor co-expression. Our approach allows for delivery of IL-21 as well as sustained PD-1 blockade with molecules that remain stable *in vivo* over prolonged periods. Using *in vitro* assays, we show that priming in the presence of an PD-1 × IL-21 fusion protein leads to enhanced cytotoxicity and effector cytokine production in antigen-specific CD8 + T cells. Moreover, in a mouse model of cancer, we demonstrate that when dosed into an anti-PD-1 mAb refractory tumor model, fusion proteins can engage tumor specific CD8+ cells to provide superior tumor control in a manner which is superior to an antagonist anti-PD-1 mAb monotherapy. Collectively our data demonstrate that this approach can harness orthogonal pathways, by antagonizing the PD-1/L1 inhibitory pathway whilst activating IL-21 cytokine signaling in a targeted manner to augment CD8+ T cell cytolytic effector function.

A significant advantage of our approach is that it allows for concentration of cytokine in a spatially restricted manner and activation of cytokine signaling in a specific population of T cells, namely PD-1 expressing cells. In addition, we show that a highly attenuated IL-21 mutein variant (R9E:R76A) has prolonged exposures and improved serum half-life as compared to recombinant free IL-21 cytokine, allowing for a longer duration between treatment cycles and a more simplified clinical trial design (33). Delivery of IL-21 cytokine to T cells as opposed to other IL-21R expressing cells including myeloid cells can overcome immune suppression associated with activation of STAT3 signaling in these cells (17). In summary, these preclinical data support the testing of these molecules across a wide range of cancer indications, including T cell infiltrated and/or PD-L1 expressing tumors previously refractory to PD-1/L1 inhibitors. Our data suggest a fusion protein approach can overcome the current limitations of these inhibitors and may extend the activity of this class of molecules in previously refractory cancer indications.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Charles River Laboratories IACUC The Institutional Animal Care and Use Committee of Amgen (IACUC).

AUTHOR CONTRIBUTIONS

KA conceived and designed the project. NA conducted the computational analysis and designed mutations. JP guided the design pharmacokinetic studies and interpreted the data. SS, GS, DO, AS, and AL performed *in vitro* experiments and interpreted the data. JD guided the design of *in vivo* mouse studies. JC conducted *in vivo* mouse studies and interpreted the data. SC designed and performed *in vivo* mouse studies and interpreted the data. RC designed binding studies and interpreted the data. MY, RL, BF, and MD were involved in the design, cloning, expression, and purification of protein reagents. KA wrote the manuscript, with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00832/full#supplementary-material

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Tolerizing Strategies for the Treatment of Autoimmune Diseases: From *ex vivo* to *in vivo* Strategies

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Autoimmune diseases such as multiple sclerosis (MS), type I diabetes (T1D), inflammatory bowel diseases (IBD), and rheumatoid arthritis (RA) are chronic, incurable, incapacitating and at times even lethal conditions. Worldwide, millions of people are affected, predominantly women, and their number is steadily increasing. Currently, autoimmune patients require lifelong immunosuppressive therapy, often accompanied by severe adverse side effects and risks. Targeting the fundamental cause of autoimmunity, which is the loss of tolerance to self- or innocuous antigens, may be achieved via various mechanisms. Recently, tolerance-inducing cellular therapies, such as tolerogenic dendritic cells (tolDCs) and regulatory T cells (Tregs), have gained considerable interest. Their safety has already been evaluated in patients with MS, arthritis, T1D, and Crohn's disease, and clinical trials are underway to confirm their safety and therapeutic potential. Cell-based therapies are inevitably expensive and time-consuming, requiring laborious *ex vivo* manufacturing. Therefore, direct *in vivo* targeting of tolerogenic cell types offers an attractive alternative, and several strategies are being explored. Type I IFN was the first disease-modifying therapy approved for MS patients, and approaches to endogenously induce IFN in autoimmune diseases are being pursued vigorously. We here review and discuss tolerogenic cellular therapies and targeted *in vivo* tolerance approaches and propose a novel strategy for cell-specific delivery of type I IFN signaling to a cell type of choice.

Keywords: autoimmunity, dendritic cells, tolerance, type-I-IFN, tolerogenic dendritic cells, pDC, cDC

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TOLERANCE-INDUCING CELLS

Dendritic cells (DC) are best known for their antigen (Ag) processing and presenting functions, driving immunological responses directed against pathogens and malignant cells. Nevertheless, they are also crucial for coordinating immunological tolerance and preventing autoimmunity. Several types of DCs exist: conventional (cDC), plasmacytoid (pDC), and monocyte-derived (moDC). They all originate from $CD34^+$ hematopoietic progenitor cells in the bone marrow. For a long time, it was generally believed that differentiation via macrophage/DC progenitors (MDC) gave rise to either the monocyte/macrophage lineage or to common DC progenitors (CDP), which further differentiated into either pDCs or pre-cDCs (1, 2). Recently, however, single-cell analysis formally demonstrated that pDCs do not develop from myeloid but from lymphoid progenitors, indicating an early divergence of pDC and myeloid-derived cDC lineages (3). Monocyte-derived DCs (moDCs) differentiate from monocytes during inflammation, induced by cytokines such as GM-CSF, IL-4, and TNF.

Vaccination with or induction of tolerogenic DCs (tolDC) could constitute a powerful therapy for autoimmune diseases. As many studies do not separate cDCs from moDCs in their analysis, it is not unequivocally clear whether endogenous moDCs also contribute to immune tolerance, besides cDCs and pDCs (4). In humans, DC research and experimental therapy by necessity focuses on moDCs, generated *ex vivo* by cytokine treatment of peripheral blood monocytes obtained via leukapheresis. To what extent these artificially produced moDCs really resemble primary endogenous DCs is not clear. It has been shown that they share some functional features with cDCs, but their overall gene expression patterns are much closer to monocytes than to any DC subset (2).

In mice, pDCs have been identified to be crucial for tolerance in several autoimmune disease models. Although most cells in the body are able to produce type I interferon (IFN-I), pDCs have been termed natural IFN-I-producing cells because of their unique adaptations to nucleic acid-sensing, which result in rapid and robust IFN-I release. Nevertheless, their *in vivo* contribution to antiviral and other infectious immune responses is probably less crucial than originally assumed (5). In Experimental Autoimmune Encephalomyelitis (EAE, the mouse model for MS), αPDCA1-induced pDC depletion or selective abrogation of MHCII expression on pDCs exacerbates EAE from the onset on (6, 7), while cDC depletion in cDC11-iDTR mice worsens disease during the later effector phase (8). In addition, PDCA1$^+$ or SiglecH$^+$ CD11c^{int} pDCs differentiated *ex vivo* from bone marrow-derived cells induce recovery (9). Also in acute graft-versus-host-disease (GvHD, induced via allogeneic bone marrow transplantation) and cardiac allograft models (10, 11), as well as in RA, asthma, T1D, and even atherosclerosis (12–15), pDCs have well-demonstrated tolerogenic functions, predominantly dependent on IDO (indoleamine-2,3-dioxygenase) and resulting in Treg induction and expansion (2, 4, 16).

In addition, type 1 and/or type 2 conventional DCs (CD8$^+$ DEC205$^+$ cDC1, C11b$^+$ DCIR2$^+$ cDC2) may also contribute to peripheral Treg differentiation and/or expansion and hence tolerance, both in homeostasis (17) and in certain autoimmune diseases such as EAE (4, 18–20). Also, in GvHD, host CD11c$^+$ cDCs were shown not to be required for the induction of disease but rather to restrict alloreactive T cell expansion (21). In addition, protection against GvHD was recently revealed to involve the tolerogenic action of both CD8$^+$ cDC1 and CD11b$^+$ cDC2 (22, 23). In T1D, however, there is preclinical evidence for a predominant tolerogenic role for DCIR2$^+$ cDC2, driving Treg expansion rather than differentiation (2, 24).

The mechanism by which tolDC instigate tolerance clearly involves the induction and expansion of Tregs. These are CD4$^+$ Foxp3$^+$ and may be generated in the thymus as natural Tregs or induced in the periphery as iTregs. Tregs are known to exert their immunosuppressive effect mainly via IL-10 and TGFβ production, which have well-established inhibitory effects on effector T cells (Teff) and positive effects on regulatory B cells (Bregs). Furthermore, Tregs may spread peripheral tolerance by generating tolDC from DC progenitors or by maintaining cDCs in an immature state (25–28). While most studies have reported no differences in the numbers of circulating Tregs in MS, RA, or T1D patients, defects in Treg phenotype and suppressive and migratory capacity have been demonstrated (29–32). Bregs represent a small population of B lymphocytes participating in immune suppression. Many of the different B cells with suppressive characteristics are CD5$^+$ (33). A particular population, which is CD5$^+$ CD1d$^+$, are very potent producers of IL-10 and are hence often referred to as B10 lymphocytes. Like Tregs, Bregs perform their regulatory functions primarily via the production of IL-10 and TGFβ as well as IL-35 (34). They have recently been recognized as very important immune modulators in various autoimmune diseases, including MS, RA, T1D, and IBD, offering novel potential strategies for therapeutic interventions (35–39).

EX VIVO TOLERANCE-INDUCING CELLULAR THERAPIES IN CLINICAL TRIALS

The number of patients suffering from autoimmune diseases and allergies is rising dramatically (40). To avoid or dampen the aberrant harmful immune response against a specific (auto)Ag, immunological tolerance is warranted. Dampening of the immune response is also required for people receiving organ or stem cell transplants. This is currently achieved by administering "all-purpose" immunosuppressive drugs, which cause both immediate and late side effects, including increased risk for life-threatening infections and malignancies.

With the identification of tolerance-inducing cell types, significant progress has recently been made in the manufacturing and usage of tolerance-inducing cells. However, as these autologous cells are generated and manipulated *ex vivo*, this personalized therapy is very laborious and expensive, with many challenges, pitfalls, and safety issues (41, 42). In addition, it remains unclear whether these artificially engineered cells adequately resemble their endogenous primary counterparts *in vivo*.

Amongst the different tolerogenic cell types, the application of tolDC is most advanced (**Figure 1A**). The first clinical study on tolDC therapy was performed in 2011 in adult T1D patients. Since then, phase I and II clinical trials have been conducted for T1D, RA, Crohn's disease, and MS. TolDC therapy is safe and shows signs of causing clinical improvement in certain patients (43, 44). In addition, tolDCs have also proven their immune dampening and thus protective potential in animal models of transplantation and allergic asthma, and clinical trials in kidney and liver transplant recipients are being set up (45–47). Once injected, tolDCs are expected to induce tolerance through various mechanisms, including the induction of Tregs and Bregs, and the stimulation of autoreactive T cell anergy and apoptosis (43).

Not only tolDC but also other myeloid regulatory/immunosuppressive cell types are currently being explored, including immature myeloid-derived suppressor cells (MDSC) and activation-induced regulatory macrophages (Mregs) (48, 49). The latter are monocytes matured through adherence to plastic surfaces and exposure to various serum factors and/or cytokines and acting through IDO, IL-10, and

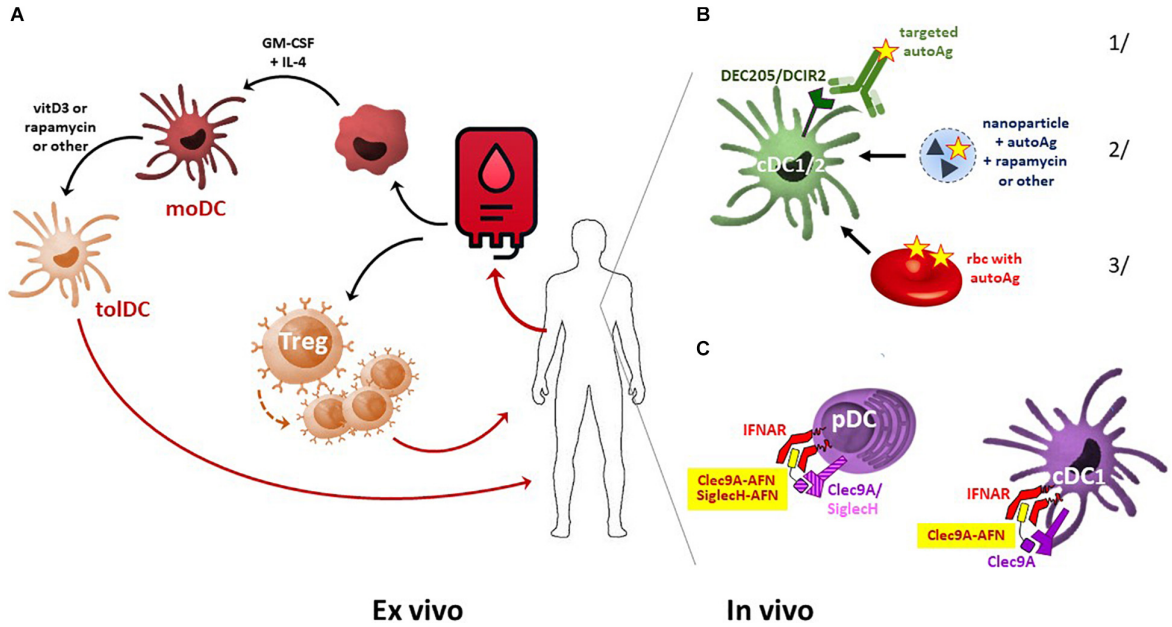

FIGURE 1 | Comparison of *ex vivo*- and *in vivo*-generated tolDC. **(A)** For cellular tolDC therapy, monocytes are isolated from patient-derived peripheral blood, driven into moDC development using cytokine therapy, and subsequently tolerized by immunosuppressive agents such as vitamin D3 or rapamycin. These autologous tolDCs are then used for patient-specific treatment. From peripheral blood, Tregs may also be sorted and further expanded *ex vivo*. Once injected back into the patient, these Tregs dampen the immune system via multiple pathways, including the suppression of DC maturation. **(B)** *In vivo* induction of tolDC may be achieved by several approaches. Examples include delivering autoAg to DCs specifically via 1/antibody-mediated targeting of DC surface markers, 2/encapsulation in nanoparticles, microparticles, or liposomes, loaded (or not) with an immunosuppressive agent, or 3/infusion of Ag-carrying erythrocytes that will be cleared via phagocytosis predominantly by DCs and macrophages. **(C)** Delivery of self-Ag may add to disease development in a pro-inflammatory microenvironment, and autoAg patterns are not always uniform or stable over time. Alternatively, selective delivery of IFN-I signaling in pDC and cDC1 by AcTaferons (AFNs, targeted using SiglecH or Clec9A single-domain antibodies) may safely and cell-specifically induce systemic tolerance.

TGFβ. *In vitro*, human Mregs are capable of deleting activated T cells, suppressing T-cell proliferation, and driving naive T cells to become Tregs, and the protective capacity of donor-derived Mregs is being explored in kidney transplant recipients (50).

The *ex vivo* expansion of autologous blood-derived Tregs has also been a clinical focus for inducing tolerance in autoimmune diseases such as GvHD, T1D, MS, Crohn's disease, SLE, autoimmune hepatitis and uveitis, and in kidney transplant patients (43, 47). The outcomes of the completed trials indicated that Treg therapy is feasible and safe. However, like tolDC generation, this strategy requires personalized, complex, and expensive manufacturing processes. In addition, current techniques lack specificity as they expand polyclonal rather than Ag-specific Tregs and also carry the risk of expanding so-called unstable Tregs that may lose their tolerogenic function and undergo transformation to pathogenic T cells, exacerbating disease.

Still another cell type with tolerogenic capacity is the mesenchymal stromal cell (MSC) population, a non-hematopoietic, multipotent, and self-renewing population found in bone marrow as well as in other tissues such as umbilical cord, muscle, and adipose tissue, that has a proven potential to modulate anti-inflammatory monocytes and macrophages, DCs, B and T lymphocytes, and NK cells (51, 52). Clinical trials with *ex vivo*-expanded MSC have been successfully conducted, showing good tolerability and therapeutic potential in MS, RA, Crohn's disease, SLE, and GvHD. A significant advantage of MSC therapy over other cell-based tolerogenic therapies is their lack of MHC expression, expanding the source of cells from autologous to allogeneic. In addition, MSC sources are multiple, including umbilical cord tissue and lipo-aspirate (43).

GENERATION AND MECHANISMS OF TOLDC

Human autologous tolDCs are generated *ex vivo*, starting from peripheral blood monocytes obtained via leukapheresis and cell sorting (**Figure 1A**). After culturing in the presence of GM-CSF and IL-4 to drive their development into moDCs, tolerization is usually achieved by treating with immunomodulatory agents such as vitamin D3, rapamycin, dexamethasone, corticosteroids, or specific cytokines (IL-10, TGFβ, IFNβ) (**Figure 1A**). Depending on the nature of the tolerizing protocol, the exact mechanisms involved in inducing systemic tolerance may diverge (53). Interestingly, whatever the tolerization protocol, this *ex vivo* approach will automatically lead to the generation of moDCs, which have gene expression patterns closer to monocytes than to DCs (2). As already mentioned, the endogenous DC subset that is typically found to be involved and necessary for protection in various autoimmune diseases is primarily the pDCs. In view of the recent finding that pDCs are not myeloid-derived, as

was thought for decades, but are rather lymphoid-derived (3), the efficacy/efficiency of myeloid-derived moDCs as tolDCs can be questioned. Also in the cancer immunotherapy field, the disappointing performance of moDCs has been suggested to be due to an intrinsic lack of biological potency as compared to endogenous cDCs (54).

Both pDCs and cDCs induce tolerance by promoting immunosuppressive Treg differentiation and function. Important endogenous signaling agents for these processes include IL-10, TGFβ, retinoic acid (RA), and kynurenine produced by IDO (55). IDO is not expressed constitutively in DCs and requires induction by various pro- and anti-inflammatory mediators such as IFNs and TGFβ. Tolerance induced by IDO may even result in so-called "infectious" tolerance, spreading from one cell to another due to kynurenines acting as activating ligands for the aryl hydrocarbon receptor (AhR) and as such for the induction of IDO expression in other cells (56). In addition, IDO activity results in tryptophan catabolism and hence metabolic stress, negatively affecting Teff proliferation and survival. Furthermore, pDCs and cDCs can also induce peripheral tolerance by inducing Teff cell anergy, i.e. functional inactivation due to checkpoint inhibitions (18).

TYPE I INTERFERON IN AUTOIMMUNE DISEASES

At least 80 different forms of autoimmune diseases exist. Together, approximately 8% of the world's population suffers from an autoimmune disease, and prevalence is sharply increasing (40). Autoimmune diseases mainly afflict women (>80%), strike at the prime of life, and cause significant debilitation, morbidity, and even mortality. In many of the most prevalent autoimmune diseases, various roles for type I IFN (IFN-I) have been described. Type I IFNs consist of a large group of structurally similar cytokines and include 13–14 subtypes of IFNα along with IFNβ, IFNε, IFNκ, IFNω, IFNδ, IFNζ, and IFNτ, all signaling through the same receptor composed of two subunits, IFNAR1 and IFNAR2. In most autoimmune models, both pathogenic and protective roles have been described, primarily for IFNα and/or IFNβ, probably depending on the disease state and the microenvironment. In general, it is important to realize that cytokines such as IFNα and IFNβ may exert different functions depending on the inflammatory context, location, and activation status of the responsive cell types.

IFNβ was the first disease-modifying therapy approved for the treatment of MS patients. Despite its therapeutic use for more than a quarter of a century, its precise mode(s) of action and specific target cells are still not completely understood. Most MS patients benefit from IFNβ therapy, but some exhibit no response or even a worsening (57). This may be due to differential effects on different cell types. In addition, side effects due to systemic toxicity preclude dose escalation and trigger therapy drop out.

In mice, triggering endogenous IFN-I release via TLR therapies can protect against IBD induced by DSS or IL-10 deficiency (58–60). Next to the activation of TLR7 or TLR9, endogenous IFN-I may also be induced by the ER-associated protein STING (stimulator of IFN genes), activated by cyclic dinucleotides. STING was shown to be important for maintaining intestinal homeostasis, and it was hence proposed that modulating the STING pathway may be of benefit in IBD (61). However, endogenous STING signaling induces both pro- and anti-inflammatory cytokines, and indeed, STING agonists were recently shown to exacerbate colitis (62). Collectively, these reports suggest that the beneficial effect of IFN-I in IBD is probably local and/or cell-specific.

From experiments performed in diabetic mice and rats, the role of IFN-I in T1D pathogenesis was originally believed to be beneficial (16). Later, this protective role was questioned, as IFNα produced by pancreatic β-cells or by pDC was shown to hasten murine diabetes progression (63, 64), and a detrimental role for pDC-derived IFNα in the initiation of T1D was eventually concluded from experiments in NOD mice (65). Nevertheless, ingestion of low-dose IFNα preserved β-cell function in recent-onset T1D patients (66), and additional clinical trials have since shown protective effects of ingested IFNα in patients suffering from MS (67).

Also in arthritis models and human RA, various roles for IFN-I have been proposed, ranging from detrimental to protective. Several experiments performed in both mice and monkeys, as well as pilot studies in RA patients, clearly suggest clinically meaningful improvement due to IFNβ treatment (68). Interestingly, a protective role has also been demonstrated for pDC, and clinical trials with tolDC are ongoing (47, 69). The controversial results using systemic IFN-I could possibly indicate local and/or cell-specific differential effects of IFN-I.

Using a murine GvHD model, TLR7 agonists were found to protect IFNAR1-dependently, involving the tolerogenic action of cDCs and increased Treg responses (22). Furthermore, selective activation of IFN-I pathways prior to hematopoietic stem cell transplantation was shown to be dependent on IFN-I signaling in $CD11c^+$ DC, reducing their ability to stimulate allogeneic T cells (23).

In addition, it has been suggested that the lack of IFN-I secretion by pDCs contributes to the development of a TH2 response in allergic asthma and that treatment of chronic allergic diseases with IFN-I may be a promising way to induce tolerance (70).

STRATEGIES TO INDUCE TOLDC *IN VIVO*

Ex vivo-generated tolDCs are well-tolerized and may have protective effects, but they also have several disadvantages, as they represent a personalized, laborious, and expensive therapy that raises many safety and economic concerns. To overcome these limitations, new approaches to induce tolDCs *in vivo* are being vigorously explored (**Table 1**). Examples include selective self-Ag targeting toward the DC receptor DEC205 before or after EAE immunization (19, 71, 72), or toward the pDC receptor SiglecH or the cDC2 receptor DCIR2 before EAE immunization, to promote immunological tolerance (20, 71, 73)

TABLE 1 | Summary of approaches to inducing tolDC *in vivo*.

Experimental mode	Tolerizing approach	Cells targeted	Specificity	Timing	Results	References
EAE (PLP, SJL)	PLP Ag delivery	cDC1 + LC	DEC205 Ab	−10 d/−15 d	Prevent and reduce disease severity ∼prevent Ag response + T suppressive mechanism	[72]
EAE (MOG, C57Bl/6)	MOG Ag delivery	pDC	SiglecH Ab	−1 d	Delay onset and reduce disease ∼prevent Ag response, no effect Tregs	[73]
EAE (MOG, C57Bl/6)	NP + peptide + AhR ligand	APC	Non-specific	d0	Suppress disease progression ∼expand Tregs	[79]
EAE (MOG, C57Bl/6)	MOG Ag delivery	cDC1 + LC cDC1 migratory cDC1 resident cDC2	DEC205 Ab Langerin Ab Treml4 Ab DCIR2 Ab	−14 d −14 d −14 d −14 d	Prevent and reduce disease Prevent and reduce disease Prevention/reduction minimal Prevention/reduction minimal ∼Protection correlates with Treg generation	[71]
EAE (spinal cord, C57Bl/6)	MOG Ag delivery	cDC1 + LC	DEC205 scFv	−7 and −3 d or +7 and 11 d	Prevent disease TGFβ-dependently ∼reduce IL-17 & IFNγ in $CD4^+$ T cells ∼induce $TGF\beta^+$ capacity in DCs	[19]
EAE (MOG, C57Bl/6)	NP + peptide + IL-10	APC	Non-specific	−30 and −15d or +8 and 22d	Reduce disease severity ∼reduced IFNγ and IL-17 by splenic T cells	[77]
EAE (PLP, SJL)	Highly negative MP	Inflammatory mono	via MARCO	+7 d; start relapse	Prevent disease; relapse ∼reduced inflammation CNS (especially DC)	[81]
EAE (MOG, C57Bl/6; MPB, B10.PL)	NP + Ag	Liver sinus EC accumulation	Selected NP	+1 d; +8−12 d	Prevent; reduce disease score ∼TGFβ and Treg dependently	[90]
EAE (PLP, SJL)	NP + Ag + rapamycin	APC	Non-specific	−14 and −21 d; +13 d	Prevent disease; relapse ∼prevent Ag response, induce Treg/Breg	[78]
EAE (MOG, PLP, C57Bl/6)	NP + MHCII-MOG-peptide or MHCII-PLP-peptide	APC	Non-specific	+14 d; +21 d	Reduce disease severity ∼Ag-experienced Teff→Tr1 (APC-dependent) ∼formation and expansion Bregs	[80]
EAE (MOG, C57Bl/6)	MOG Ag decorated rbc	Phagocytes	Non-specific	7 d/+5 d/+11 d	Prevent or cure disease ∼Th17 decrease in CNS	[83]
EAE (PLP, SJL)	NP + peptide + rapamycin	APC	Non-specific	+14 d = peak	Prevent relapse ∼prevent Ag response + expand Tregs	[74]
EAE (PLP, SJL)	PLP Ag delivery	$CD11b^+$ cDC2	DCIR2 Ab	−10d	Prevent and reduce disease ∼reduce pathogenic T_H + expand Tregs	[20]

(Continued)

TABLE 1 | Continued

Experimental mode	Tolerizing approach	Cells targeted	Specificity	Timing	Results	References
EAE (MOG, C57Bl/6)	Engineered IFNα delivery	pDC, cDC1	SiglecH sdAb or Clec9A sdAb	+7 d/+12 d	Delay onset, reduce disease progression ∼IDO/TGFβ^{+} pDC; IL-10/TGFβ^{+} Treg and Bregs	[89]
Diabetes (p31-T transfer)	p31 peptide delivery	rbc → uptake DC	Ly76 scFv → non-specific	+8 h till 7 d	Prevent hyperglycemia ∼deletion transferred diabetogenic T cells	[91]
Diabetes (NOD/BDC2.5)	NP + IAg7 (NOD/BDC2.5 MHCII)	APC	Non-specific	10 wk of age	Prevent incidence ∼Ag-experienced Teff → Tr1 (APC-dependent) ∼formation and expansion Bregs	[80]
Diabetes (NOD+BDC2.5-T)	BDC2.5 peptide delivery	cDC2 cDC1	DCIR2 Ab DEC205 Ab	−1 and 0 d −1 and 0 d	Delay diabetes induction ∼ T cell apoptosis no effect	[24]
Diabetes (NOD mice)	NP + Ins Ag + AhR ligand	APC	Non-specific	8 wk of age	Reduce disease development ∼tolDC, Treg differentiation	[92]
Diabetes (NOD mice)	Ins Ag decorated rbc	Phagocytes	Non-specific	10 wk of age	80% protection	[83]
IBD (DSS, C57Bl/6)	Highly negative MP	Inflammatory mono	via MARCO	+1−6 d	Suppress disease score ∼reduced inflammation /colon	[81]
Inflammatory arthritis	liposomes + Ag + NFκB⊥	cDC, pDC, mf	Non-specific	+6 d	Reduce disease severity ∼induction Tregs, suppression Teffs	[76]
Arthritis (CIA)	NP + MHCII-collagen peptide	APC	Non-specific	@130% swelling	Reduce disease severity ∼Ag-experienced Teff → Tr1 (APC-dependent) ∼formation and expansion Bregs	[80]
Skin transplantation	MHC-I monomer delivery	cDC2	DCIR2 Ab	−14 d	Long term allograft survival if CD8-depleted	[93]
Liver transplantation	NP + tacrolimus	APC	Non-specific	+4 till 10 d	Prolong allograft survival	[94]
Heart transplantation	Targeted NP + αCD3	HEV, LN	MECA79 Ab	−1 d till 3 d	Prolong allograft survival ∼Treg dependent	[95]
Heart transplantation	HDL-NP + rapamycin	Myeloid cells (mf)	Non-specific	+6 d	Long term allograft survival ∼Mreg dependent	[96]

Ab, antibody; Ag, antigen; AhR, Aryl hydrocarbon receptor; APC, antigen-presenting cell; cDC, conventional dendritic cell; d, day; EC, endothelial cell; HEV, high endothelial venules; Ins, insulin; LC, Langerhans cell; LN, lymph node; mf, macrophages; MOG, myelin oligodendrocyte glycoprotein; mono, monocytes; MP, microparticles; NP, nanoparticles; pDC, plasmacytoid dendritic cell; PLP, proteolipid protein; rbc, red blood cells; sdAb, single-domain antibody; scFv, single-chain variable fragment; wk, week.

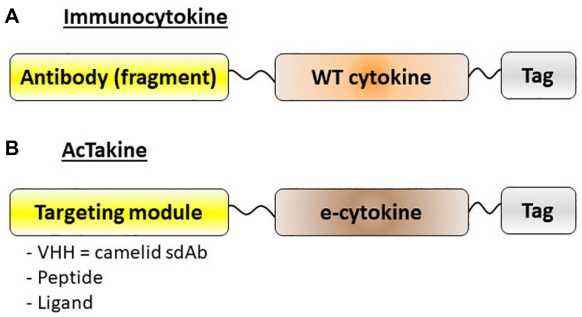

FIGURE 2 | Schematic representation of immunocytokines and AcTakines. **(A)** Immunocytokines are typically engineered by coupling a wild-type (WT) cytokine to a targeting module, usually an antibody or antibody fragment. **(B)** AcTakines consist of a mutated (engineered) e-cytokine with reduced cognate receptor affinity, coupled C-terminally via a 20xGGS linker to a targeting moiety. In general, a camelid-derived single domain antibody (sdAb = VHH) is used for the latter, although peptides or ligands can also be employed. For purification purposes, AcTakines are decorated with a C-terminal affinity tag.

(**Figure 1B**). Other successful approaches include injection of autoAg-containing nano- or microparticles or liposomes. These are taken up via phagocytosis or endocytosis, predominantly by antigen-presenting cells (APC, including DCs and myeloid cells), and disease-relevant peptides or proteins can be co-encapsulated with immunosuppressive agents such as rapamycin, IL-10, NFκB inhibitors, or AhR ligands (74–79). Most of these reported studies were performed in EAE and T1D models, but their efficacy has also been illustrated in other autoimmune diseases such as arthritis and IBD (76, 80, 81) and in various transplantation models (82) (**Table 1**). In addition, transfusion with autoAg-decorated red blood cells (rbc) (**Figure 1B**), which are known to be preferentially phagocytosed by DCs and macrophages, has recently proven its efficacy in both EAE and NOD diabetic mice (83). Importantly, maturation or activation signals for DCs, present under inflammatory conditions, may abrogate the tolerogenic protection conveyed by autoAg delivery (20), and as such endogenous inflammation could turn a self-Ag-based DC tolerizing therapy into one further exacerbating disease (44). In addition, self-Ag patterns are not always uniform or stable during disease development and progression.

Not only DCs and myeloid cells are being targeted; efforts are also underway to selectively stimulate the *in vivo* expansion of Tregs. So far, most trials have concentrated on the use of low-dose IL-2 to achieve this, since IL-2 is crucial for T-cell proliferation and its receptor is most abundant on natural Tregs, but an optimal and long-lasting regime has not yet been found and agreed upon (30, 43). Furthermore, no pharmacological approaches are currently available to selectively expand autoAg- or disease-specific Tregs *in vivo*.

Given the possible protective role of IFN-I in autoimmune diseases, especially in MS, we decided to apply our targeted AcTaferons in the EAE model. AcTaferons (AFNs) are IFN-I based AcTakines (Activity-on-Target cytokines). Basically, AcTakines are a novel class of engineered immunocytokines, the key difference between AcTakines and immunocytokines being the exclusive use of mutant (engineered) e-cytokines with severely reduced receptor affinity instead of wild-type (WT) cytokines (84) (**Figure 2**). While immunocytokines, where WT cytokines are fused to targeting antibodies or antibody fragments, can still bind with great affinity to their ubiquitous receptors while traveling through the body, causing residual side effects and their systemic removal [the so-called "sink" effect (85)], AcTakines cannot signal when administered systemically except in those cells that express a surface molecule specifically recognized by the targeting moiety linked to the mutant cytokine. As a result, AcTakines do not cause the multiple toxic side effects usually accompanying cytokine therapies. In addition, they provide unique research tools for dissecting the *in vivo* cell-specific functions of pleiotropic cytokines under normal or pathophysiological conditions. Thanks to a convenient "plug-and-play" assembly of modular building blocks, AcTakines can be engineered easily by coupling various mono- or multimeric e-cytokines to targeting moieties such as camelid-derived single-domain antibodies (sdAb, VHH), peptide motifs specifically recognized by receptor isoforms, or ligands interacting with their cell-specific cognate receptors. During recent years, we have successfully and safely employed various cell-specific AcTakines as potential anti-tumor therapies (86–88). Recently, we also obtained evidence in EAE that DC-targeted AFN can be used to specifically target IFN-I signaling to DCs as an *in vivo* method to induce tolerance (89) (**Figure 1C**). Systemic tolerance was evident in pDCs (increased numbers and an enhanced tolerogenic signature including IDO and TGFβ production) as well as in Tregs and Bregs, both of which produced significantly more IL-10 and TGFβ. Interestingly, pDC targeting was superior to cDC1 targeting during early progression of EAE, but cDC1 targeting later during disease progression significantly added to the protection. In contrast to therapy with autologous *ex vivo*-generated moDCs derived from a cell lineage that may not be optimal (myeloid), AFNs deliver the IFN-I signaling potential specifically to endogenous pDCs and cDC1s *in vivo*. Furthermore, cell-specific targeting not only limits the possibility of aspecific toxic side effects but also avoids signaling in unwanted cell types. The relevance of the latter becomes clear when comparing the protective capacities of untargeted WT IFN-I with CD8- or DC-targeted AFN. While WT IFN-I can delay disease onset and DC-targeted AFN provides profound protection, CD8-targeted AFN worsens disease (89). This strategy still leaves many more options open, such as selective targeting to B lymphocytes, specific myeloid subsets, and more.

AUTHOR CONTRIBUTIONS

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

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IL-15 in the Combination Immunotherapy of Cancer

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We completed clinical trials of rhIL-15 by bolus, subcutaneous, and continuous intravenous infusions (CIV). IL-15 administered by CIV at 2 mcg/kg/day yielded a 38-fold increase in 10- day number of circulating NK cells, a 358-fold increase in CD56^{bright} NK cells and a 5.8-fold increase in CD8 T cells. However, IL-15 preparations administered as monotherapy were ineffective, due to actions of immunological checkpoints and due to the lack of tumor specific targeting by NK cells. To circumvent checkpoints, trials of IL-15 in combination with other anticancer agents were initiated. Tumor-bearing mice receiving IL-15 with antibodies to CTLA-4 and PD-L1 manifested marked prolongation of survival compared to mice receiving IL-15 with either agent alone. In translation, a phase I trial was initiated involving IL-15 (rhIL-15), nivolumab and ipilimumab in patients with malignancy (NCT03388632). In rhesus macaques CIV IL-15 at 20 μg/kg/day for 10 days led to an 80-fold increase in number of circulating effector memory CD8 T cells. However, administration of γc cytokines such as IL-15 led to paralysis/depression of CD4 T-cells that was mediated through transient expression of SOCS3 that inhibited the STAT5 signaling pathway. This lost CD4 helper role could be restored alternatively by CD40 agonists. In the TRAMP-C2 prostate tumor model the combination of IL-15 with agonistic anti-CD40 produced additive effects in terms of numbers of TRAMP-C2 tumor specific Spas/SCNC/9H tetramer positive CD8 T cells expressed and tumor responses. A clinical trial is being initiated for patients with cancer using an intralesional anti-CD40 in combination with CIV rhIL-15. To translate IL-15-mediated increases in NK cells, we investigated combination therapy of IL-15 with anticancer monoclonal antibodies including rituximab in mouse models of EL-4 lymphoma transfected with human CD20 and with alemtuzumab (CAMPATH-1H) in a xenograft model of adult T cell leukemia (ATL). IL-15 enhanced the ADCC and therapeutic efficacy of both antibodies. These results provided the scientific basis for trials of IL-15 combined with alemtuzumab (anti-CD52) for patients with ATL (NCT02689453), with obinutuzumab (anti-CD20) for patients with CLL (NCT03759184), and with avelumab (anti-PD-L1) in patients with T-cell lymphoma (NCT03905135) and renal cancer (NCT04150562). In the first trial, there was elimination of circulating ATL and CLL leukemic cells in select patients.

Keywords: interleukin-15, natural killer cells, CD8 T cells, immunotherapy of cancer, immunological checkpoints

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INTRODUCTION

The goal of immunotherapy is to direct the host immune system to attack patients' cancer (1, 2). Clinical trials initially focused on efforts to enhance immune responses using the stimulatory cytokines IFNα or IL-2 (2). High-dose IL-2 was approved by the FDA for the treatment of patients with metastatic melanoma and metastatic renal carcinoma (3) but caused severe systemic toxicity, including capillary leak syndrome, hypotension, hypoxia, and oliguric renal failure. These problems prompted the investigation transition to IL-15 in an effort to obtain the benefits of IL-2 but with fewer adverse events (AEs) (2).

IL-15 was identified by our group and by Grabstein in culture supernatants from HUT102 and Cv1/EBNA cell lines that stimulated proliferation of the cytokine dependent T-cell line CTLL-2 (4–6). IL-15 is a 14–15 kDa 4 alpha-helix-bundle family cytokine family member that stimulates the generation of NK, NKT, gamma delta, IL/C1, intraepithelial lymphocytes, innate cells expressing CD103+ CD56+ CD44+ and memory CD8 T cells (2, 7–17). Some IL-15 regulation of protein production occurs at the level of transcription; however, most control is at translation (18). Type I and II interferons, CD40 ligation, and Toll-like receptor stimuli stimulate transcription (19). IL-15 translation is impeded by multiple 5′-untranslated region (UTR) AUG sequences, a long signal peptide and a negative regulatory element in the coding sequence C-terminus (19). IL-15 mRNA is expressed by many tissues. However, IL-15 protein is largely limited to dendritic cells, macrophages, and monocytes (20). IL-15 signals through a heterotrimeric receptor that is composed of the common gamma chain (γc) subunit (CD132) shared with IL-2, IL-4, IL-7, IL-9, and IL-21; the beta chain (βc) subunit (IL-2/IL-15R, CD122) shared with the IL-2 receptor and a private IL-15 specific alpha subunit IL-15Rα (CD215) (2, 8, 20, 21). IL-15 binding to the IL-2/IL-15Rβ/γc heterodimeric receptor induces JAK1 activation that phosphorylates STAT3 via the beta chain, and JAK3 activation that phosphorylates STAT5 via the gamma chain (20–25).

IL-15, like IL-2, stimulated proliferation of T cells, induced generation of cytotoxic lymphocytes and memory phenotype CD8 T cells, and stimulated proliferation and maintenance of natural killer (NK) cells (2, 8, 20). In contrast to IL-2, IL-15 did not mediate activation-induced cell death (AICD), did not consistently activate Tregs and caused less capillary leak syndrome (2, 8, 26). IL-2 is a promiscuously secreted molecule, whereas IL-15 is locally secreted in small quantities where membrane-bound IL-15 induces signals at an immunological synapse (27–32). IL-15 and IL-15Rα co-expressed by monocytes and DCs become associated on cell surfaces where IL-15 is presented *in trans* to NK and CD8 memory T-cells (27–32). In addition, IL-15 cis presentation is required for optimal NK-cell activation in lipopolysaccharide-mediated inflammatory conditions (33).

Although IL-2 stimulates immune responses directed at cancer cells, it also suppresses immune responses by maintenance of $CD25^+$ Foxp3 T-regulatory cells and by participation in AICD (34–37).

Efficacy was observed with IL-15 in multiple murine immunotherapy trials including the syngeneic TRAMP (transgene adenocarcinoma mouse prostate) -C2 prostatic cancer, Pme1-1, B16 melanoma, MC38 and CT26 colon carcinoma models suggesting that IL-15 might be more effective than IL-2 in cancer therapy (38–40). Ten-day 20 mcg/kg/day administration of IL-15 to rhesus macaques by continuous infusion (CIV) was associated with an 80–100 fold increase in the number of circulating effector memory CD8 T cells (41, 42). To translate the observation of the effect of IL-15 on NK cells and CD8 cells, we have completed first-in-human trials of rhIL-15 by bolus, subcutaneous and continuous intravenous infusions (CIV) (2, 43–45). However, IL-15 administered as monotherapy was ineffective, likely due to the actions of immunological checkpoints (2). To circumvent such checkpoints, trials of IL-15 in combination with other anticancer agents have been initiated and are a major focus of this review.

CLINICAL TRIALS USING IL-15 IN THE TREATMENT OF CANCER

We initiated a first-in-human phase I trial of recombinant *Escherichia coli* produced IL-15 administered by IV bolus daily for 12 days to patients with metastatic malignancy (2, 43) (**Table 1**). The initial dose of 3 μg/kg/day was too toxic with patients developing grade 3 thrombocytopenia and hypotension, and doses of 1.0 and 0.3 μg/kg/day were added (2, 43). All patients at the 0.3 μg/kg dose level received 12 doses without dose-limiting toxicity (DLT). With the 3 μg/kg dose level as assessed by flow cytometry there was a 10-fold increase in the circulating NK numbers, a 3-fold increase in the number of CD4 cells and an 8-fold increase in the number of CD8 T cells. Stable disease was the best response. Inflammatory cytokines IL-6 and IFN-γ were markedly elevated (50-fold), a phenomenon which coincided with acute clinical toxicities of fever, chills and blood pressure changes. To reduce toxicity by reducing C_{max} excess, mediated cytokine release, and macrophage activation syndrome, two additional clinical trials were initiated, one by subcutaneous, and another by continuous intravenous infusion (2, 44, 45).

In the subcutaneous rhIL-15 trial in refractory solid tumor cancer patients' therapy consisted of daily (Monday–Friday) subcutaneous injections of rhIL-15 for 2 consecutive weeks (44). Nineteen patients were treated with rhIL-15. Among 19 patients treated there were two serious events: grade 2 pancreatitis, grade 3 cardiac chest pain, hypotension, and elevated troponin. No objective responses were observed. Treatment induced a 3-fold increase in the number of circulating CD8 T cells, a 10.8-fold expansion of circulating NK cells, and a 39.7-fold increase in $CD56^{bright}$ cells.

In an additional trial 27 patients were treated for 10 days by continuous intravenous infusion with rhIL-15; with 2.0 μg/kg/day identified as the MTD (45). There were eight serious adverse events including: papilledema, uveitis, pneumonitis, duodenal erosions, two bleeding events, and two deaths, one likely due to drug-related gastrointestinal ischemia (2, 45). Limited reduction in tumor volume was observed in several

TABLE 1 | IL-15 Clinical trials in patients with metastatic malignancy.

IL-15 agent	MTD or expansion dose/dosing schedule	Study population	Serious and notable adverse events	Maximum fold increase in total NK cells at MTD	Maximum fold increase in CD56 bright NK cells	Maximum fold increase in CD8 T cells	Best clinical Response	References
E. coli rhIL-15	0.3 μg/kg/d bolus i.v. 12 consecutive days	18 patients with malignant melanoma or renal cell cancer	Grade 3 hypotension Grade 3 thrombocytopenia Grade 3 ALT, AST elevations	2–3	3–4	3	Stable disease (5 patients had 10–30% decrease in marker lesions and 2 disappearance of lung lesions)	Conlon et al. (43) National Cancer Institute, NIH
E. coli rhIL-15	2 μg/kg/d CIV for 10 days	27 patients with metastatic solid tumors	2 deaths (one due to gastrointestinal ischemia and one due to disease progression) Grade 3 bleeding Grade 3 papilledema Grade 3 uveitis Grade 3 hepatic encephalopathy	38	358	5.8	Stable disease	Conlon et al. (45) National Cancer Institute, NIH
E. coli rhIL-15	2 μg/kg/d SC days 1–5, 8–12	19 patients with advanced solid tumors	Grade 2 pancreatitis Grade 3 cardiac/chest pain	10.8	39.7	3.3	Stable disease	Miller et al. (44) Minnesota Cancer Center
ALT-803	10 μg/kg IV or SC weekly for 4 weeks	33 patients with hematological malignancies	2 deaths (one due to sepsis, one due to intracranial hemorrhage) Grade 4 sepsis Grade 2 pemphigus	8	8	2	1 CR, 1 PR, 3 SD	Romee et al. (46) Minnesota Cancer Center
ALT-803	20 μg/kg SC 4 consecutive weeks every 6 weeks	21 patients 11 IV, 13 SC with solid tumors	Grade 4 congestive heart failure Grade 4 neutropenia Injection site reaction	3.3	6.3	6.3	No PR or CR	Margolin et al. (47) Fred Hutchinson Cancer Center

CIV, continuous intravenous infusion; d, day; IL-15, interleukin 15; IV, intravenous; Kg, kilogram; NA, not available; SC, subcutaneous; CR, complete response; PR, partial response; SD, stable disease; CRS, cytokine release syndrome.

patients, but stable disease per RECIST 1.1 criteria was the best response noted (45). In this trial, the IL-15 C_{max} was at 48 h, followed by a decline of serum IL-15 concentrations during the infusions to 8% of the maximum level by days 8–10 of infusion. This decline may reflect IL-15-mediated induction of the number of IL-15 receptor-bearing cells with an increase in the number of IL-2/IL-15Rβ (CD122) receptors per cell acting as a sink binding the infused rhIL-15. There was a mean 5.8-fold increase in the number of circulating CD8 T cells, a 38-fold increase in the total of NK cells and a 358-fold increase in $CD56^{bright}$ NK cells (2, 45).

Studying purified NK cells *in vitro* Felices et al. (48) suggested that continuous treatment with IL-15 exhausts purified NK cells resulting in decreased viability and a cell cycle arrest gene expression pattern. Furthermore, they propose that their findings should inform IL-15 dosing strategies (2, 48). Our studies with IL-15 *in vivo* by CIV to humans do not support these conclusions. The proliferation rates of different subsets of NK cells 2 days after the termination of 10-day IL-15 CIV assayed by Ki-67 were over 90% (45, 49). The cytolytic capacities were very effective for both $CD56^{dim}$ and $CD56^{bright}$ NK subsets. At the maximum NK level 2 days following the termination of IL-15 CIV the antibody-dependent cellular cytotoxicity (ADCC) assayed with CD20 antibody-coated Raji cells, natural cytotoxicity to K-562 cells mediated by NKp30, NKp46, and MICA/NKG2D mediated cytotoxicity was exceptionally effective (2, 49). These observations on the effects of IL-15 on NK subsets do not support the hypothesis that such strategies would be associated with NK-cell exhaustion but rather support the view that after 10-day rhIL-15 CIV NK cells remain effective.

A major challenge with rhIL-15 is that it has a short *in vivo* survival. Therefore, an array of alternative IL-15 agents associated with IL-15Rα were introduced clinically (50–61) (**Figure 1**). These included an IL-15N72D mutein with a 4-5-fold increase in biological activity, heterodimeric mammalian IL-15/IL-15Rα (hetIL-15) (51–54) a heterodimer consisting of IL-15 and IL-15Rα (51–54), the RLI, a fusion protein consisting of IL-15 linked to the cytokine binding (sushi) domain of IL-15Rα (59, 60), RLI-anti-CD20 and RLI-CD20 which are RLI linked to anti-CD20 or GD2 (55, 61), ALT-803 a mutated (N72D) IL-15 linked to the sushi domain of IL-15Rα that is fused to an IgG-Fc fragment to increase *in vivo* survival (55–57), the ALT-803 scaffold fused to 4-single chains of rituximab, a tumor-targeting monoclonal antibody (2, 58). ALT-803 was administered to 33 patients with hematological malignancies via IV or SC once weekly for 4 doses and pharmacokinetic analysis showed prolonged serum concentrations following SC compared to IV infusion (46). There were 2 deaths–one due to sepsis, and one intracranial hemorrhage purported to be unrelated to ALT-803. Administrations of hetIL-15 or ALT-803 by subcutaneous injection produced concentric injection site reactions up to 30 cm in diameter erythematous plaques that were associated with infiltration of CD56+ $NKp46^{-}$ γδ T-cells. This and other systemic AEs with ALT-803 and hetIL-15 precluded further increases in doses of IL-15 bearing agents. When the maximum fold increases in the number of circulating NK cells with different agents and dosing schedules were compared, rhIL-15 administered by bolus infusion at the MTD (0.3 mcg/kg/day) yielded a 2–3 fold increase in NK cells (43) (**Table 1**). rhIL-15 administered subcutaneously at the expansion dose of 2 mcg/kg/day on days 1–5, and 8–12 was associated with a 10.8-fold increase in total circulating NK cells and a 39.7-fold increase in $CD56^{bright}$ NK cells (44). The ALT-803 mutant at 10 mcg/kg/week elicited an 8-fold increase in NK cells (46). rhIL-15 by CIV at 2 mcg/kg/day for 10 days resulted in the greatest increase with a 38-fold increase in circulating total NK cells and a 358-fold increase in $CD56^{bright}$ NK cells (45).

Novel approaches with IL-15 are being developed to yield the desired pharmacokinetics of IL-15 plus IL-15Rα with one dosing per week along with maximal increases in NK and CD8 T cells provided by CIV rhIL-15. PEGylated IL-15 to prolong survival is being developed. Furthermore, a long-acting rhIL-15 depo for enhanced cancer immunotherapy is being developed, with IL-15 mixed with an aqueous solution of PLGA-PEG, a copolymer that is in solution at room temperature but transitions into a hydrogel at body temperature (Tan and Waldmann unpublished observations).

IL-15 IN COMBINATION THERAPY

IL-15 and Haploidentical Natural Killer Cell Therapy for Advanced Acute Myeloid Leukemia

Although rhIL-15 by CIV yielded dramatic augmentation in the number of circulating NK cells, it will have to be used in combination with other anticancer agents due to the inhibitory actions of immunological checkpoints and the lack of tumor specific targeting by NK cells (**Tables 2**, **3**). A major challenge in IL-15 immunotherapy is finding a combination of drugs with new mechanisms of action that improve the outcome achieved with the existing standard of care and simultaneously result in fewer toxic effects for patients. Forty-two patients with refractory acute myeloid leukemia received intravenous (IV) (NCT01385423) or subcutaneous (SC) (NCT02395822) recombinant human IL-15 (rhIL-15) after lymphodepleting chemotherapy and haploidentical NK cell infusions (75). Escalating doses of rhIL-15 (0.3–1.0 mcg/kg) were given on 12 consecutive days in a phase I trial to 26 patients. Subcutaneous IL-15 at 2.0 mcg/kg was administered in a phase II trial to 16 patients. With the IV dosing and dose level 3 (1 mcg/kg) dose-limiting toxicity consisting of grade 4 pulmonary toxicity (diffuse alveolar hemorrhage) in one patient and prolonged neutropenia (beyond 42 days) in 2 of 4 patients at this dose were observed. In the subsequent phase II trial using 2 mcg/kg SC of rhIL-15 there was a previously unreported cytokine release syndrome (CRS) observed in 56% of patients with concurrent neurological toxicity in 5 of 9 patients that was responsive to steroid and tocilizumab treatment. Eight of 25 evaluable patients receiving IV IL-15 had a response with 6 CRs and 2 CRis. The overall response to SC IL-15 was 6 of 15 patients with a CR in one and CRi in 5 (75). Thus, haploidentical NK cell infusions given with rhIL-15 achieved remissions in about 35% of patients with refractory acute myeloid leukemia.

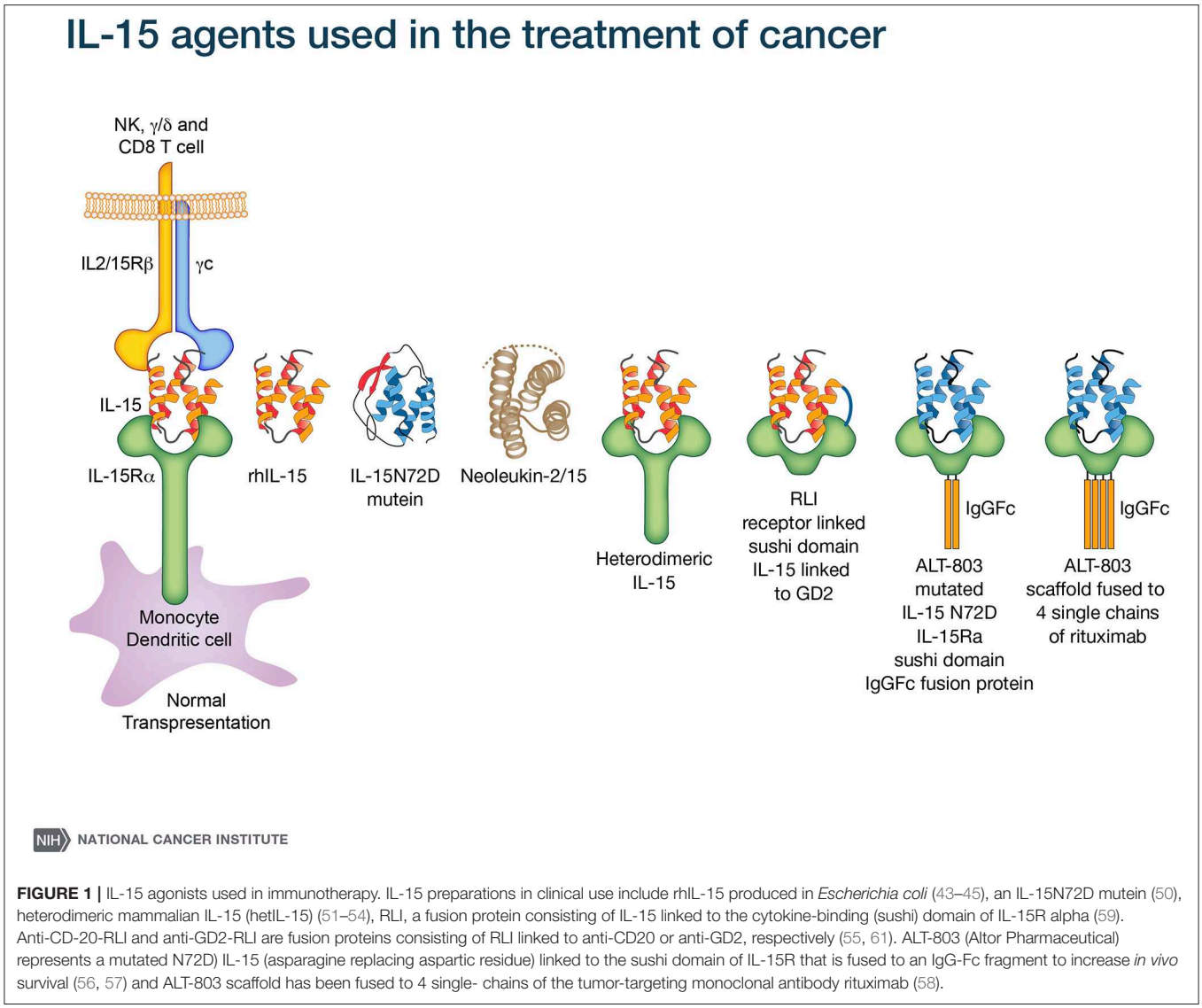

FIGURE 1 | IL-15 agonists used in immunotherapy. IL-15 preparations in clinical use include rhIL-15 produced in *Escherichia coli* (43–45), an IL-15N72D mutein (50), heterodimeric mammalian IL-15 (hetIL-15) (51–54), RLI, a fusion protein consisting of IL-15 linked to the cytokine-binding (sushi) domain of IL-15R alpha (59). Anti-CD-20-RLI and anti-GD2-RLI are fusion proteins consisting of RLI linked to anti-CD20 or anti-GD2, respectively (55, 61). ALT-803 (Altor Pharmaceutical) represents a mutated N72D) IL-15 (asparagine replacing aspartic residue) linked to the sushi domain of IL-15R that is fused to an IgG-Fc fragment to increase *in vivo* survival (56, 57) and ALT-803 scaffold has been fused to 4 single- chains of the tumor-targeting monoclonal antibody rituximab (58).

Combination of IL-15 Plus Agonistic Anti-CD40

In rhesus macaques IL-15 by CIV at 20 mcg/kg/day for 10 days led to 80 to 100-fold increases in circulating effector memory CD8 T cells (41). Furthermore, rhIL-15 by CIV to patients with metastatic malignancy led to a mean 5.8-fold increase in the number of circulating CD8+, MHC class II^{+} cells (2, 45). However, this effect was not associated with evidence that the CD8 T cells manifested antitumor activity nor did it provide anticancer efficacy. In terms of CD8 T-cell function, γc cytokines such as IL-15 induced immunoregulatory SOCS checkpoint agents. IL-15 increased the expression of CIS, a checkpoint of NK cell mediated tumor immunity as well as SOCS1 that attenuates IL-15 receptor signaling by CD8+ CD44^{hi} memory T lymphocytes (77, 78). Furthermore, Sckisel et al. demonstrated that administration of gamma cytokines such as IL-2 and IL-15 led to paralytic depression of CD4 T cell that was mediated through transient expression of SOCS3 that inhibited STAT5B signaling (2, 79). This paralysis of CD4 helper T-cell activity inhibited the generation of tumor-specific CD8 T cells. It was demonstrated that CD4 helper cells' role could be provided by CD40 agonists (67, 68, 80–82). We showed in the TRAMP-C2 murine syngeneic tumor model that treatment with either an agonistic anti-CD40 antibody alone or IL-15 prolonged animal survival, however the combination of agonistic anti-CD40 with IL-15 produced markedly additive effects when compared with either agent alone (68). Neither agonistic anti-CD40 nor IL-15 augmented the number of tumor-specific CD8 T cells, whereas administration of the combination of IL-15 with an agonistic anti-CD40 antibody was associated with a 10-fold increase in the number of SPAS/SCNC 9H tetramer positive anti-TRAMP-C2 tumor specific CD8 T cells (68). Examination of this tumor system was extended by evaluating TRAMP-C2 administered on each flank

TABLE 2 | Preclinical studies of IL-15 IN combination immunotherapy and cancer.

IL-15 agent	Combination agent	Study	Best response to IL-15	Reference
rhIL-15	Rituximab	Mouse graft EL4 transfected with human CD20	Prolongation of survival increase in ADCC	Zhang et al. (62) National Cancer Institute, NIH
rhIL-15	Alemtuzumab	Mouse xenograft with ATL cell line	Prolongation of survival increase in ADCC	Zhang et al. (62) National Cancer Institute, NIH
RLI		HCT human colon carcinoma B16F10	NK mediated reduce tumor growth overcoming limited effect of IL-15	Bessard et al. (60)
RLI anti-GD2		SC EL4, metastatic N x S2 Neuroblastoma	Better murine survival than anti-G2D or RLI alone	Vincent et al. (61)
RLI anti-CD20		Human B cell lymphoma in SCID mice	Prolonged survival of mice beyond that of RLI or anti-CD20 alone	Vincent et al. (55)
rhIL-15	Cetuximab	Triple negative breast cancer cell line EGFR expression Hbbr with KRAS mutation 50:1 effector target	Increase in TBMC, ADCC against cell lines from 28 to 34% without IL-15 to 71% with this increase in NK expression and activation of receptors	Roberti et al. (63) Centro de Investigaciones Oncológicas, Buenos Aires, Argentina
ALT-803	Anti-CD20	Primary human B cell lymphoma and B cell lines. Two human NK xenografts in NOD/SCID mice	Significant increase in degranulation, IFNα production, decrease in tumor cells, and ADCC by human NK cells against B cell lymphoma. Increase mouse survival.	Rosario et al. (64) Johns Hopkins Medicine, Baltimore, Maryland
IL-15	Rituximab	CLL cells $\gamma c^{-/-}$ mice	Enhanced cytotoxicity against CLL cells with overcome TGFβ mediated immunosuppression.	Moga et al. (65) Department of Immunology Hospital Santa Creu i Sant Pau, Barcelona, Spain
ALT-803 fused to Rituximab 2B8T2M		B-cell lymphoma cells xenograft to SCID/NOD mice	2B8T2M better cytokines, better survival of mice with xenografts better depletion of B cells in monkeys.	Liu et al. (66) Alto BioScience Corp, Miramar, Florida
rhIL-15 0.25 mcg/day daily 5x/week for 4 weeks	Anti-CD40	TRAMP-C2 graft in mice	Prolongation of survival of mice with xenograft. Development of tumor specific CD8 T cells.	Zhang et al. (67, 68) National Cancer Institute, NIH
ALT-803	Anti-gp75, TA99 anti-PD-L1	Mice bearing B16F10	Prolong survival through activation of NK cells and expansion of $CD8^{+}CD44^{high}$ T cells. Addition of anti-PD-L1 further increases antitumor activity.	Chen et al. (56) University of South Carolina Medical School
TriKE bispecific NK cell engaged against CD16 modified IL-15 crosslinker.		Cr51 release degranulation vs. carcinoma cell lines.	TriKE with IL-15 when compared to BiKE without IL-15 showed enhanced ADCC with improved activation and survival of NK cells.	Schmohl et al. (58) University of Minnesota Medical School
rhIL-15	Anti-CTLA-4 Anti-PD-L1	Mouse TRAMP-C2 prostate, CT26 colon carcinoma models.	Simultaneous inhibition of two regulatory 7-cell inhibitory checkpoints enhanced IL-15 efficacy in murine tumor models.	Yu et al. (69, 70) National Cancer Institute, NIH
IL-15 sIL-15Rα/Fc	Anti-PD-1	HT-29 xenograft in NOD/SCID mice.	Tumor growth inhibition.	Zhao et al. (71) Shanghai University
IL-15/IL-15Rα armed oncolytic virus	Anti-PD-1	MC38 colon mouse carcinoma or ID8 ovarian cancer models.	CD8 T cell mediated by IL-15 armed oncolytic virus. Antitumor immunity was dramatically improved by addition of anti-PD-1	Kowalsky et al. (72) University of Pittsburgh School of Medicine
ALT-803 (N-803)	Anti-PD-L1	4TI Triple negative breast and MC38 colon tumor bearing mice	ALT-803 enhanced anti-PD-L1 antitumor efficacy by increasing CD8 T cell effector function.	Knudson et al. (73) National Cancer Institute, NIH

with anti-CD40 administered intratumorally in one flank tumor and IL-15 administered systemically. In this model there was an abscopal effect obtained with reduction in the size of the tumor not injected with anti-CD40 beyond that mediated by IL-15 alone. A clinical trial is being initiated that utilizes an optimized intralesional anti-CD40 antibody in combination with CIV rhIL-15 (83, 84).

Agents to Relieve IL-15 Induced Checkpoints on the Immune System to Augment IL-15 Action

IL-15 augments the expression of immune checkpoints TIGIT, TIM3, IL-10, as well as the expression of PD-1 on CD8 T cells (85, 86). Furthermore, IL-15 is required for the expression of a negative regulatory lymphocyte population that expresses

TABLE 3 | Clinical trials of IL-15 in combination immunotherapy of cancer.

IL-15 and combination agent	MTD or expansion dose/dosing schedule	Study population	Serious and notable adverse event	Maximum fold increase of NK cells	Best clinical response	References
ALT-803 + nivolumab	20 μg/kg ALT-803 sc combination with IV nivolumab every 2 weeks	21 patients with metastatic non-small cell lung cancer	Grade 3 myocardial infarction. Injection site reaction.	3	6 PR, 10 SD	Wrangle et al. (74) Medical University of South Carolina, Health Hollings Cancer Center
E. coli rhIL-15 with haploidentical NK cell infusion	IL-15, 1.0 mcg/kg for 12 consecutive days IV with haploidentical NK cell infusion 2.0 mcg sc for 10 doses	42 patients: 26 IV and 16 sc with refractory acute myeloid leukemia	One patient died with cerebral infarct intracranial aspergilloma. 9 of 16 sc patients had CRS including fever, hypotension and in 5 of 9 concurrent neurotoxicity including one Grade 5.	NA	Of 15 IV patients: 6 CR and 2 Cri.	Cooley et al. (75) Masonic Cancer Center, University of Minnesota
E. coli rhIL-15 alemtuzumab	IL-15 sc Mon-Fri 0.5, 1.0, 2.0 mcg/kg/day for 2 weeks, followed by alemtuzumab 3, 10, 30 mcg/kg/day	8 patients with mature T cell malignancy	None	15	PR, CR elimination of leukemic T cells in each of 7 patients studied with leukemia	Miljkovic et al. (76) National Cancer Institute, NIH

CIV, continuous intravenous infusion; d, day; IL-15, interleukin 15; IV, intravenous; Kg, kilogram; NA, not available; SC, subcutaneous; CR, complete response; PR, partial response; SD, stable disease; CRS, cytokine release syndrome.

CD122+ CD8+ (87). The combination of anti-PD-L1 with ALT-803 yielded additivity in murine tumor models (71–73, 88). Furthermore, trans-signaling with the RLI human IL-15 linked to the human IL-15Rα sushi domain augmented effector memory CD8 T-cell responses and enhanced antitumor activity of the PD-1 agonist (72). ALT-803 in combination with nivolumab in individuals with non-small cell lung cancer was associated with an objective response in 6 of 21 patients (74). To address checkpoints, we administered IL-15 in combination with antibodies to PD-L1 and cytotoxic lymphocyte antigen-4 (CTLA-4) in the CT26 and MC38 colon carcinoma and TRAMP-C2 prostatic cancer syngeneic tumor models (2, 69, 70). In these models IL-15 alone provided modest antitumor activity. The addition of either PD-L1 or CTLA-4 in association with IL-15 did not augment its efficacy. However, tumor-bearing mice receiving the combination of both anti-checkpoint antibodies with IL-15 manifested a significant prolongation of survival. In translation of this observation, a phase I trial in patients with refractory cancers has been initiated that involves rhIL-15 in combination with nivolumab and ipilimumab (NCT03388632).

IL-15 in Combination Therapy With Anticancer Monoclonal Antibodies

As noted above, rhIL-15 administration led to dramatic increases in the number of activated NK cells, however such increases alone were not sufficient to produce antitumor efficacy probably because most tumors express self MHC class I molecules that interact with KIRs or NKG2A/CD94 that inhibit NK-effector function (2). Furthermore, there is lack of tumor cell identification and specific targeting by NK cells. The combination of IL-15 with tumor specific monoclonal antibodies has shown efficacy with a number of anticancer antibodies (**Tables 2**, **3**) (55, 56, 58, 62, 64, 72). In preclinical trials IL-15 preparations have been reported to be of value in combination with *in vivo* administered anticancer monoclonal antibodies. In particular, IL-15 increased ADCC and antitumor activity when administered with anti-gp75, with B16F10 tumors (56) and with anti-CD20 with B-cell lymphomas (65). The combination of IL-15 with anti-PD1 or anti-PD-L-1 was more effective than with the individual agents alone (62, 69, 72, 75). In addition, an engineered fusion protein involving a soluble form of human IL-15Rα sushi with an antibody demonstrated antitumor responses (58). Furthermore, there was an enhanced ADCC and anti-breast cancer efficacy of cetuximab with a chimeric protein encompassing human IL-15 (62). We also investigated a combination therapy that involves IL-15 with rituximab in a syngeneic mouse model of EL4 transfected with human CD20 and with alemtuzumab (CAMPATH-1H) in a xenograft model of human adult T-cell leukemia (ATL) (69). IL-15 enhanced the therapeutic efficacy of both antibodies. This efficacy was dramatically reduced in FcRγ$^{-/-}$ mice suggesting that IL-15 increased the ADCC of the anticancer monoclonal antibodies. Both NK cells and macrophages were critical elements of interacting effectors involved in the augmented ADCC and augmented therapeutic responses (71). Following interaction with macrophages there was induction of expression of FcRγIV critical for ADCC by the NK cells (2, 74). These results provided the scientific

basis for a phase I trial of IL-15 combined with alemtuzumab (anti-CD52) for patients with ATL (NCT02689453) (2). Trials have also been initiated in patients with chronic lymphocytic leukemia with obinutuzumab in combination with rhL-15 (NCT03759184) and IL-15 with avelumab (anti-PD-L1) in patients with mature T-cell lymphoma (NCT03905135) and renal cell cancer (NCT04150562), and IL-15 with mogamulizumab (anti-CCR4, NCT04185220) in patients with ATL and cutaneous T-cell lymphoma.

CONCLUSIONS

Despite their dramatic augmentation of NK cells and CD8 T cells, all IL-15 preparations administered as monotherapy in solid tumor patients with cancer have been ineffective probably due to counter-regulatory immunologic processes. In particular, there was inhibition of NK action by interaction of KIRs and NKG2A with self-class I MHC. There was parallel inhibition of CD8 T cells stimulated by IL-15 due to the induction of SOCS3 in CD4 helper T cells, thereby yielding "helpless" CD8 T cells (79). Furthermore, IL-15 induced checkpoints TIGIT, TIM3, IL-10, and PD-1 on CD8 T cells (85, 86). To circumvent these checkpoints combination trials that involve IL-15 with multiple anticancer agents are being performed. Our combination therapeutic trials include IL-15 with intralesional agonistic anti-CD40 to yield tumor specific CD8 T cells, IL-15 with the checkpoint inhibitors, anti-CTLA-4 and anti-PD-L1, and especially IL-15 with cancer directed monoclonal antibodies to increase their ADCC and anticancer efficacy. It is hoped with the use of these combination therapies that IL-15 will take a prominent role in the treatment of patients with metastatic malignancy.

AUTHOR CONTRIBUTIONS

TW designed, wrote, and edited the manuscript. SD, MM, and KC provided critical comments, concepts, and insights. All authors read and approved the manuscript prior to submission.

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frontiers in Immunology

REVIEW
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Modulating Cytokine Production via Select Packaging and Secretion From Extracellular Vesicles

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Cytokines are soluble factors that play vital roles in systemic function due to their ability to initiate and mediate cell-to-cell communication. Another important mechanism of intercellular communication that has gained significant attention in the past 10 years is the release of extracellular vesicles (EVs). EVs are released by all cells during normal physiology, in states of resting and activation, as well as during disease. Accumulating evidence indicates that cytokines may be packaged into EVs, and the packaging of cytokines into EVs, along with their ultimate secretion, may also be regulated by cytokines. Importantly, the repertoire of biomolecules packaged into EVs is shaped by the biological state of the cell (resting vs. activated and healthy vs. disease) and the EV biogenesis pathway involved, thus providing mechanisms by which EV packaging and secretion may be modulated. Given the critical role of cytokines in driving acute and chronic inflammatory and autoimmune diseases, as well as their role in establishing the tumor immune microenvironment, in this review, we will focus on these disease settings and summarize recent progress and mechanisms by which cytokines may be packaged within and modulated by EVs, as a therapeutic option for regulating innate and adaptive immunity.

Keywords: extracellular vesicles, biogenesis, secretion, trafficking, therapeutics, intercellular, communication

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INTRODUCTION

Intercellular communication is an essential biological feature that is mediated through (1) cell-cell contact, (2) soluble factors (cytokines, growth factors, hormones, neurotransmitters) and (3) the more recently discovered extracellular vesicles (EVs) that carry cytosolic, nuclear and cell-surface proteins, lipids, nucleotides, microRNA, and metabolites (1, 2). These three mechanisms of intercellular communication help to ensure that homeostasis is maintained in a biological system and that the system can respond appropriately to conditions of stress and disease. Conversely, dysregulation of any of these mechanisms of intercellular communication may promote altered physiology leading to disease.

Cytokines are small, non-structural proteins with low molecular weights that are synthesized and secreted by immune cells: macrophages, B and T cells, dendritic cells, neutrophils, mast cells, as well as endothelial, epithelial, fibroblasts and stromal cells, as a mechanism to communicate with each other (3). As soluble factors, they are largely responsible for promoting and regulating an immune response by acting on receptors at the cell membrane. This results in the downstream

regulation of signaling molecules that stimulate cells toward sites of inflammation, infection, and trauma (4). Thus, cytokines have significant roles in a variety of functions including cell activation, differentiation, proliferation, trafficking, inflammation, and tumorigenesis that affect every organ system in the body. Their pleiotropic function(s) as intercellular messengers allows them to act at the site they are produced (autocrine), on nearby cells (paracrine), or on distant cells and tissues (endocrine), which also enables them to be self-regulating (4, 5). Cytokines act as extracellular ligands for specific membrane receptors present on responsive target cells and thus must possess a high affinity for each other. The high affinity helps to explain why cytokines can exert their biological effects in picomolar concentrations (4). As such, it is not surprising that multiple mechanisms have evolved that allow for the fine-tuning of cytokine secretion that enables an effective but limited response. This level of control is necessary in order to prevent excessive and/or dysregulated release that could drive acute and chronic inflammatory and autoimmune diseases (5, 6). As a result, it is important to understand the secretory (exocytic) pathways and endocytic compartments involved in cytokine transport, along with the regulatory molecules and cellular machinery that determine the levels and timing of cytokine release [reviewed in (5–9)]. Although cytokines are considered soluble factors, recent data indicate that they can also function as membrane proteins and be packed and stored in secretory granules, lysosome-related organelles, or secretory lysosomes and later released at the cell surface (8). Accumulating evidence indicate that cytokines can also reach the extracellular space through EVs.

EVs are a heterogeneous collection of small membrane-bound organelles that are naturally released from all cells [recently reviewed in (10)]. Originally, they were described as small vesicles that selectively remove excess and unnecessary components of cells in order to maintain homeostasis. However, subsequent studies over the past 10 years reveal that EVs play an important and targeted, functional role in cell-to-cell communication (11). Studies from multiple labs show that the packaging of cellular components within EVs are determined, in part, by the cell type they are secreted from and the physiologic status of the parental cell (12–15); the latter involving mechanisms that can be manipulated to potentially alter the cellular components within EVs and the secretion of EVs.

Based on biochemical and microscopic characterization of EVs, they can be broadly separated into two classes—exosomes and microvesicles—that are primarily distinguished by the mechanisms of biogenesis, as well as size (11). Details of the mechanisms of EV biogenesis have recently been reviewed (10). Briefly, exosomes range in size between ∼50 and 150 nm in diameter (∼100 nm on average) and arise from the endo-lysosomal trafficking pathway during the formation of multivesicular bodies (MVBs). Exosomes are released extracellularly when MVBs fuse with the plasma membrane. Microvesicles, on the other hand, are organelles generated by pathways that direct the outward budding or shedding of the plasma membrane and range in size between ∼50 nm to 1 μm. More recent data in the field of EVs indicate that these two classes also differ by the cellular components that are packaged inside, likely resulting in different biological functions (11). The unique profile of cellular components that are packaged in EVs and secreted from a cell represents a molecular, biological, and cellular code that contains information about the parental cell at the time of secretion and how the EVs may reprogram recipient, adjacent cells and tissues during normal homeostasis and disease (14). However, precise identification of the origin of EVs is made difficult by the fact that there is substantial commonality in size, external markers, and internal content between exosomes and microvesicles. As a result, it is often not possible to definitively establish the method of biogenesis of isolated EVs, underscoring the importance of clearly defining the parameters used to identify specific EV populations (16). In this review, we provide an overview of cellular states and mechanisms by which cytokines may be packaged within and their release controlled by EVs.

PACKAGING OF CYTOKINES IN EVS

While all innate immune cells have the capacity for constitutive exocytosis, their release can also occur through regulated secretory pathways [reviewed in (9, 14)]. The constitutive and induced secretion of cytokines as soluble factors provides a systemic release that helps to maintain normal homeostasis. Regulated secretion, on the other hand, provides the ability to orchestrate the rapid delivery of a concentrated amount of cytokines to a specific site in response to a specific signal (9). Recent work by Fitzgerald et al. revealed that cytokine packaging into EVs was a general biological phenomenon that occurs *in vitro*, *ex vivo* and *in vivo* from multiple cell types and tissues. Somewhat surprising, they found that all cytokines could be packaged into EVs. However, depending on the biological system and cell type, they reported that a cytokine could be released either in soluble or EV-associated form. Analysis across multiple biological systems (placental villous explants, tonsil explants, amnion explants, cervix explants, plasma, T cells, amniotic fluid, monocytes) revealed that 9 cytokines—Interleukin 6 (IL6), IL8, IL13, IL16, IP10, MCP1, MIP1α, MIP1β, and MIP3α–were more often found in soluble form. Conversely, 11 cytokines—IL2, IL4, IL12p70, IL17, IL21, IL22, IL33, IFNγ, ITAC, TGFβ, and TNFα–were found in greater levels in EVs. An interesting aspect of this study that is relevant to disease was the finding that cytokines packaged into EVs are not detected by standard cytokine assays, such as ELISA or other multiplexed immunoassays, since they are hidden from antibody detection by the EV membrane. Thus, methods to determine cytokine production from EVs will be important for our understanding of their role(s) in health and disease (12, 17).

What exactly is the biological meaning of packaging cytokines in EVs? Given that cytokines can exert their biological effects in picomolar concentrations, packaging cytokines into EVs is one mechanism whereby cytokine expression may be concentrated at the surface of other cells that might not otherwise be targeted by cytokines in soluble, circulating form. Further, EV packaging may facilitate cytokine delivery and targeting to distant cells. This could be mediated by binding of EV-surface cytokines to cells that express specific cytokine receptors. Another possibility

is that EVs protect cytokines from environmental degradation. Indeed, Fitzgerald et al. found that EV-associated cytokines were protected from trypsin digestion, as compared to soluble cytokines (12). This protection extends to cytokines bound to the surface of EVs as well, since an 189 amino acid isoform of VEGF was found to associate with heparin on the surface of small cancer-derived EVs, resulting in reduced recognition by the VEGF antibody bevacizumab (18). Interestingly, these data suggest a mechanism by which vesicle surface-bound VEGF contributes to bevacizumab resistance in cancer patients that is likely different than soluble VEGF function. Further, synovial fibroblasts from patients with rheumatoid arthritis were shown to release EVs that express membrane-associated TNF that reduces the activation-induced cell death of $CD4^+$ T cells (19). Differences in biologic function between soluble and membrane-bound cytokine receptors have been relatively well-characterized in the literature, showing that soluble receptors will often act as antagonists to membrane-bound forms (20, 21). However, comparatively little is still known regarding the different biologic functions of soluble vs. vesicle membrane-bound cytokines. As a result, the mechanism(s) by which cytokines are packaged in EVs, by internalization as vesicle cargo or expression on the vesicle surface, and how they are released from EVs, through lysis or uptake by a target cell, all contribute to the complex mechanisms of normal (healthy) and disease-related cytokine signaling.

Although the previous 5–10 years have shown rapid advancements in the field of EV research, there remain a number of unanswered questions regarding differential biological outcomes from cytokines (and other proteins) released by EVs into the microenvironment. For instance, Rana et al. reported that poly(I:C) could induce the release of both soluble and EV-secreted IL36γ from keratinocytes (22). The authors postulated that these two mechanisms of cytokine release may modulate both local and systemic immune responses to viruses and other pathogens. However, it remains unknown whether soluble and packaged IL36γ have different biological functions on target cells. Moreover, it is not currently known whether cytokine signaling in a target cell is altered dependent on how the target cell "sees" the cytokine. This lack of knowledge is partially due to the fact that multiple mechanisms exist for how target cells interact with EVs, thus adding to the complexity of our understanding of differential function [reviewed in (23)].

CELL TYPE AND PHYSIOLOGIC STATUS DETERMINE CYTOKINE PACKAGING

As alluded to above, Fitzgerald et al. recently reported that medium from cultured cells and tissue explants, as well as body fluids, contained different amounts of EVs with different levels and types of cytokines. Importantly, they found that the distribution of cytokines between soluble and EV-associated forms was largely dependent on the cellular system rather than the cytokine being secreted. For example, tissue explants that contain cells in close proximity to other cells normally found in their *in vivo* microenvironments tended to release more cytokines in soluble form than were found in T cell or monocyte suspensions or in plasma. Indeed, a greater proportion of EV-associated cytokines were found from the cells and plasma. However, upon stimulation of cells, they found that the number and pattern of cytokines packaged in EVs changed depending on the stimulus, suggesting that the packaging of cytokines in EVs is not simply the property of a particular cytokine, but rather a tightly controlled biological process. For instance, stimulation of tonsillar explants with pokeweed mitogen resulted in a drastic change in the pattern of cytokine release with a shift toward more soluble secretion rather than EV-associated secretion. In contrast, human primary monocytes stimulated with either LPS or polyI:C resulted in more EVs being secreted with different patterns of cytokines associated with EVs; distinct patterns of soluble vs. EV-associated cytokine secretion were also detected between the two stimuli (6, 12).

Stimulation of human umbilical cord blood-derived mast cells by cross-linkage of FcIgE receptors (FcεRI) induces the release of granule-associated mediators such as histamine, metabolites, and cytokines (24–26). Kandere-Grzybowska et al. found that stimulation of human mast cells with IL1 rather than FcεRI cross-linking resulted in the exclusion of IL6 from secretory granules, and instead found that IL6 was secreted in 40-80 nm vesicular structures (27). Similarly, a number of reports have been recently published showing that exosomes from the plasma of HIV-infected individuals have distinct levels and types of cytokines as compared to exosomes from healthy donors (28–30). Interestingly, in patients with diabetes, the association of specific cytokines with EVs was found to be strongly influenced by disease duration and treatment outcome (31). Altogether, these data support that EV-associated cytokine loading and secretion may be directed in a cell type- and stimuli-dependent manner.

BIOLOGICAL ACTIVITY OF EV-ASSOCIATED CYTOKINES

In the mid-1990s, EVs secreted from B cells were shown to have an immunological function in antigen presentation and as vesicles that can induce T cell responses (32–34). We now know that one of the mechanisms by which EVs elicit immunological function is that they can serve as alternate carriers for the delivery of cytokines. Immunologically, EVs maintain characteristics of the antigen presenting cell (APC) that they were derived from, exposing the extracellular domain of major histocompatibility complex (MHC) molecules at the vesicle surface. Thus, EVs released by APCs carrying surface MHC Class I and MHC Class II can directly stimulate $CD8^+$ and $CD4^+$ T cells, respectively [reviewed in (33)]. Of note, EVs are also generated from immunosuppressive APCs. For instance, autologous EVs isolated from plasma shortly after antigen (Ag) stimulation could be used to induce Ag-specific immunosuppression (35, 36). Further, EVs isolated from bronchoalveolar lavage fluid following Ag-specific exposure could be used to prevent Ag-specific allergic responses (37, 38). Last, EVs present in human breast milk and colostrum were found to increase the number of T regulatory (T_{reg}) cells and thus could be used to suppress immune responses (37). In this context, pregnancy has been shown to alleviate the severity

of some autoimmune diseases, such as rheumatoid (RA) arthritis and multiple sclerosis (MS) (39).

Given the small size of EVs, they are capable of crossing major biological barriers such as the blood-brain barrier, and thus provide interesting prospects for therapeutic packaging and regulation (40–42). It is now well-recognized that EVs have a wide range of pleiotropic functions in multiple biological processes. For example, in an *in vitro* model of cardiovascular disease, EVs isolated from TNFα-induced human vascular endothelial cells (HUVEC) were taken up by monocytes and un-induced HUVEC, promoting an inflammatory response (13, 43, 44). Hosseinkhani et al. reported a select increase in IL6, IL8, and ICAM1 levels in un-induced HUVEC after co-incubation with EVs isolated from TNFα-induced HUVEC, while THP1 cells showed an increase in ICAM1, MIP1β, CCL5, and CXCL10 levels (13). The change in THP1 inflammatory mediators by EVs led to an increase in monocyte adhesion and migratory function. Another interesting study reported that exosomes isolated from mesenchymal stem cells (MSCs) of human umbilical cord treated with interferon (IFN)γ or a combination of TGFβ plus IFNγ contained increased levels of TGFβ, IDO, IL10, and IFNγ that, when incubated with PBMCs, resulted in increased numbers of T_{regs} (45).

In HIV-positive individuals, cytokines were found to be markedly enriched in exosomes and exposure of these exosomes to purified naïve peripheral blood mononuclear cells (PBMCs) resulted in the induction of CD38 expression on naïve and central memory $CD4^+$ and $CD8^+$ T cells, likely contributing to viral propagation via activation of bystander cells (30). An independent study characterizing plasma EVs from HIV-positive individuals found increased oxidative stress markers that correlated with an IFN gene signature and immune activation (28). Another interesting immunologic function for EVs was discovered in the placenta as a mechanism to regulate immunity against the fetus during pregnancy. Holder et al. reported that macrophage-derived exosomes containing IL6 and IL8 were actively transported into the human placenta to stimulate placental cytokines (46).

EV-ASSOCIATED CYTOKINES IN AUTOIMMUNE DISEASE

Recent evidence supports that EVs can mediate immune stimulation or suppression and can drive inflammatory, autoimmune and infectious disease pathology (47–49). One of the mechanisms by which EVs can drive autoimmune disease is that they serve as carriers of pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs, respectively), as well as cytokines, autoantigens and tissue-degrading enzymes (48). Indeed, synovial EVs from patients with RA were found to contain citrullinated proteins and, in the autoimmune disease systemic lupus erythematosus (SLE), EVs could serve as autoantigens in the formation of immune complexes (50–54). In addition, cytokines, such as IL6, are highly implicated in the development and progression of multiple autoimmune diseases whose production can be regulated by EV packaging and secretion. The role of IL6 in autoimmune disease pathogenesis is due in part to its influence on $CD4^+$ T cell lineage and regulation [reviewed in (55)]. We provide examples in the above sections of how IL6 packaging and secretion in EVs can be regulated by different stimuli.

Another cytokine that contributes to autoimmune disease pathogenesis is TNFα. High levels of circulating TNFα are a major driver of RA. Interestingly, a membrane-bound form of TNFα was recently detected from individuals with osteoarthritis (19). The premise that EVs package cytokines that contribute to the amplification of an immune response was supported by work from Obregon et al. revealing the presence of large amounts of TNFα packaged into EVs derived from LPS-activated dendritic cells (DCs). These EVs also contained MHC II, CD40, CD83, TNFR1, and TNFR2 and were internalized by epithelial cells that became activated to release cytokines and chemokines such as IL8, MCP1, MIP1β, RANTES, and TNFα (56). In another related study, Zhang et al. identified a membrane bound form of TNFα on exosomes produced from synovial fibroblasts of patients with RA. These exosomes were found to activate Akt and NFκB pathways and rendered T cells resistant to undergo apoptosis; the authors proposed that this contributed to T cell-mediated pathology in RA (19). **Figure 1** provides an overview of these mechanisms through which EVs expressing TNFα modulate autoimmunity.

IL1β is a pro-inflammatory cytokine that has stimulatory effects and helps promote the differentiation of $CD4^+$ T cells into T helper 1 (Th1) and Th17 lineages, both of which are known to contribute to autoimmune disease pathogenesis (57, 58). The release of the active form of IL1β follows a finely regulated process [(59); reviewed in (60)] and we now know that EV production plays a role in the maturation process of IL1β (61–66). This is dependent on the formation of the inflammasome, a multiprotein complex of innate immunity that is also involved in the secretion and loading of proteins associated with vesicles (67, 68). Different types of stimuli have been reported to promote inflammasome activation resulting in IL1β secretion via EVs, such as extracellular ATP that serves as a strong activator of the NRLP3 inflammasome, resulting in increased release of EVs (65, 69). Another stimulus is ionic fluxes that cause membrane polarization. It has been well-established that Ca^{2+} influx causes inflammasome activation and vesicular production. Ca^{2+} influx also induces the activation of different calcium-dependent proteins involved in membrane and cytoskeletal modification, thus facilitating the release of EVs (70). Last, a non-canonical route for inflammasome activation and the maturation of IL1β involves caspase 4/5, which directly recognize intracellular LPS. Caspase 4/5-mediated activation of the inflammasome strongly induces the release of IL1β, IL18, and other EV-associated cytokines (63).

High serum levels of type I (IFNα), II (IFNγ), and III (IFNλ1) are observed in patients with SLE and have been associated with high disease activity; thus, IFNs are considered to be key molecules in the pathogenesis of SLE (71–74). Interestingly, before EVs were identified as entities with physiologic function, it was well-known that IFNs were able to affect enveloped virus budding, release, and infectivity by increasing the expression

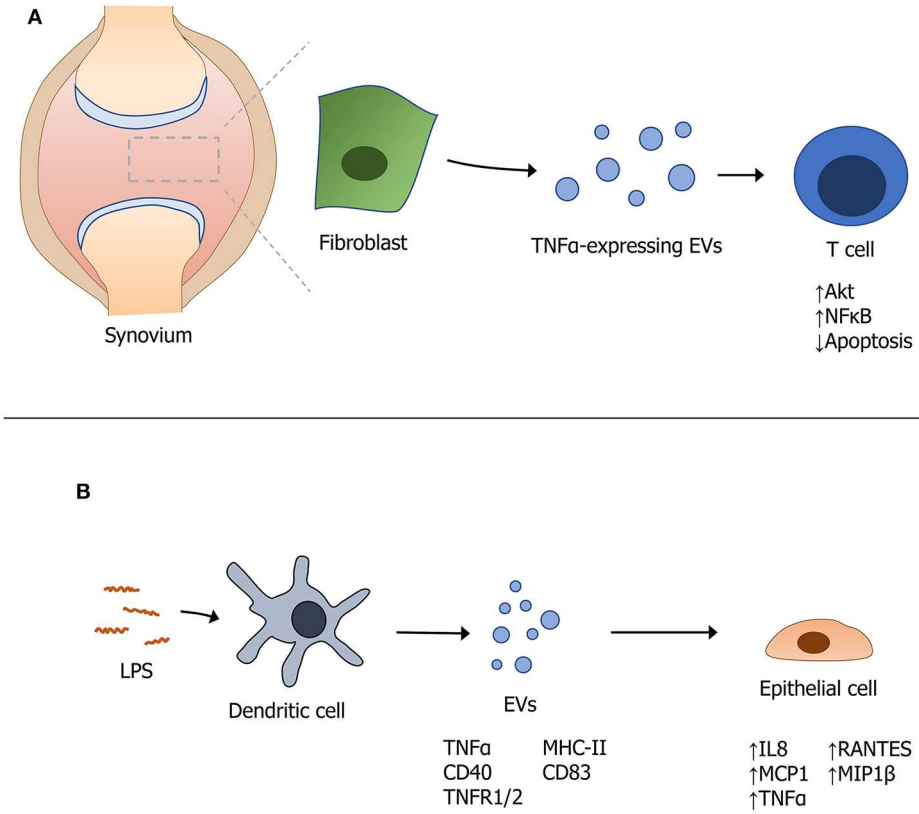

FIGURE 1 | Modulation of autoimmunity by extracellular vesicles expressing TNFα. **(A)** Fibroblast-derived EVs containing TNFα modulate T cell function in the synovium of patients with rheumatoid arthritis. **(B)** Stimulation of dendritic cells with LPS induces the packaging and secretion of specific EV-associated cytokines that themselves, induce a downstream effect(s) on cytokine production from epithelial cells.

of genes encoding restriction factors, such as ISG15 that has regulatory functions in EV packaging and secretion (75). ISG15 is an IFN stimulated gene (ISG) and an ubiquitin-like modifier (76–78). It has been identified in microvesicles and exosomes originating from TLR3 (polyI:C)-activated human brain microvascular endothelial cells (79). Importantly, ISG15 was found to ISGylate TSG101, which is a component of the ESCRT-I complex that mediates ESCRT-dependent EV secretion [reviewed in (10, 14, 80)]. Thus, not surprisingly, ISGylation was reported to influence exosome secretion. Villarroya-Beltri et al. revealed that type I IFNs trigger TSG101 modification via ISG15 that results in TSG101 degradation and impaired exosome secretion (81, 82). They reported that ISGylation of TSG101 triggers MVB co-localization with lysosomes, thus promoting the aggregation and degradation of MVB proteins, and the ultimate impairment of exosome secretion (81). Relevant to type I and II IFNs, the transcription factor interferon regulatory factor 1 (IRF1) was found to regulate select GTPases, such as Rab27a that is a key factor in EV secretion. Yang et al. found that IFNγ-induced IRF1 upregulation promoted Rab27a expression and EV secretion; conversely, knockdown of IRF1 or Rab27a resulted in reduced EV secretion (83). In addition to contributing to the regulation of EV secretion, IFNs also contribute to the packaging of its cellular components [reviewed in (6)].

EV-ASSOCIATED CYTOKINES IN CANCER

In cancer, tumor-derived EVs have been shown to play roles in immune evasion and metastatic progression (84–87). One of the first studies revealed that vaccination of mice with exosomes isolated from tumor peptide-pulsed DCs primed tumor-specific cytotoxic T cells and suppressed tumor growth in a T cell-dependent manner (88). Similarly, Seo et al. found that EVs released from activated $CD8^+$ T cells of healthy mice were capable of attenuating tumor invasion and metastasis by apoptotic depletion of mesenchymal tumor stromal cells (89). Subsequent studies of EVs secreted from melanoma and prostate cancer cells revealed that they express programmed death-ligand 1 (PD-L1) on their surface, which suppresses the function of $CD8^+$ T cells and facilitates tumor growth (90–93). The level of PD-L1 expression was found to correlate with disease stage, and was increased by IFNγ stimulation (94). Importantly, the associated suppression of $CD8^+$ T cell response by exosomal PD-L1 could be abrogated by treatment with PD-1 or PD-L1 inhibitors to induce immune-mediated reduction of tumor growth.

Most solid tumors exhibit increased release of EVs, accompanied by alterations in their composition of proteins, lipids, and genetic material (95, 96). As a result, tumor-derived

EVs have diverse effects on tumor growth, invasion, metastasis, and immune response, in part, through their modulation of cytokine production by cells of the innate and adaptive immune system (86). The complex interplay between the diverse array of cells in the tumor microenvironment and the pleiotropic factors that are secreted, is the subject of extensive current research, and our knowledge of exactly how these cells and mediators interact is incomplete. Nonetheless, it is clear that EVs promote tumor growth and progression in most solid tumors, highlighting the importance of these mediators in tumor-immune regulation.

Tumor-associated macrophages (TAMs) are major regulators of inflammation and immune response in the tumor microenvironment and are thus important targets of tumor-derived EVs. Crosstalk between tumor-derived EVs and macrophages can polarize them toward a more M2-like, pro-tumor TAM (97), which is associated with higher levels of the immunosuppressive cytokines IL10, IL4, and TGFβ. However, EVs can also promote tumor progression through an increase in pro-inflammatory functions of macrophages. Wu et al. found that exosomes secreted by gastric tumors were capable of inducing pro-inflammatory signaling in macrophages via activation of NF-κB, thereby promoting tumor growth and invasion (98). Similarly, breast cancer exosomes were found to induce macrophage-mediated secretion of the cytokines TNFα, IL6, and MCP1, which stimulate tumor progression and metastasis (99, 100). Increased IL6 production mediated by tumor-derived exosomes results in suppressed dendritic cell activity and attenuated immune response, resulting in enhanced tumor growth (101).

Tumor-derived exosomes also promote tumor growth through the stimulation of myeloid-derived suppressor cells (MDSCs), which have immunosuppressive effects in tumors. Multiple cancer types have been found to secrete exosomes containing heat shock proteins, Hsp72 and Hsp90, which activate Stat3 in MDSCs via IL6 and promote immunosuppression and tumor growth (85). Exosomes isolated from B16 melanoma tumors in mice were shown to stimulate MDSCs to produce TNFα, MCP1, and IL6 in a MyD88-dependent manner, which promotes immunosuppression, tumor growth, and metastasis (23). Not surprisingly, these same pro-inflammatory cytokines were implicated in ovarian cancer, in which exosomes isolated from the body fluids of patients induced production of IL1β, TNFα, and IL6 by THP-1 monocytes (102).

Last, tumor-derived EVs have been implicated in the development of pre-metastatic niche (PMN) formation in a variety of cancers [recently reviewed in (103)]. Results from pancreatic cancer, breast cancer, ovarian cancer, and melanoma, among others, highlights the importance of EVs in regulating intercellular communication at sites distant from the primary tumor (104). For example, in a well-characterized model of pancreatic cancer, tumors were found to secrete exosomes containing macrophage migration inhibitory factor (MIF), which induces TGFβ signaling in Kupffer cells in the liver. This resulted in increased production of fibronectin by hepatic stellate cells, creating an environment that is more permissive to metastatic colonization by tumor cells (105). In response to the hypoxic microenvironment that is present in most solid tumors, many types of cancers were found to promote endothelial growth through the release of pro-angiogenic factors, such as VEGF, that can also be packaged inside exosome (18, 106, 107). A summary of EV-mediated regulation of cytokine production by cells in the tumor microenvironment is provided in **Figure 2**.

MODULATING EV SECRETION AS A MECHANISM TO CONTROL CYTOKINE RELEASE

Although EVs are released in resting cells, stimulating events, such as cell activation, leads to increased intracellular calcium levels, resulting in cellular membrane remodeling and enhanced EV secretion (108). Pharmacologic modulation of EV output can be achieved through treatment with agents that interfere with cytoskeletal remodeling that is required for the formation of MVBs and trafficking of proteins into vesicles for their subsequent release (10, 80). Calpains are a family of calcium-dependent cysteine proteases that are important for unconventional protein secretion and inflammasome activation (65). Inhibition of calpain with a small-molecule inhibitor, such as MDL28170, blocks vesicular formation and the subsequent release of EVs (65). Given the role of caspase 4/5 in inflammasome activation and release of IL1β-associated EVs, the use of a caspase 4 inhibitor was found to block EV secretion from LPS-stimulated human macrophages (63). Treatment of cells/tissues with the microbial metabolite Manumycin A, a farnesyltransferase inhibitor, resulted in decreased EV biogenesis and secretion via modulation of ESCRT machinery (12, 109). A similar pharmacologic approach is to inhibit the formation of MVBs by inhibiting sphingomyelinase activity. Sphingomyelinases are required for the inward budding and eventual release of MVBs through an ESCRT-independent pathway. GW4869 is a neutral sphingomyelinase inhibitor that inhibits vesicle formation (110). Last, simvastatin was recently identified as an inhibitor of EV secretion based on the rationale that cholesterol is necessary for the formation of vesicle membranes. However, simvastatin's function as an HMG-CoA reductase inhibitor does not entirely explain the mechanism, as supplementation with mevalonate did not fully restore EV output to baseline levels (111). Given that different mechanisms of EV biogenesis exist, we may utilize this knowledge to selectively target (inhibit) specific populations of EVs while leaving other subsets of EVs untouched.

As our current understanding of the mechanisms that differentially regulate the packaging of cytokines in EVs from resting and activated cells expands, this knowledge may be also used to preferentially drive the packaging of distinct groups of cytokines into EVs for therapeutic use. For instance, DCs can be stimulated to secrete EVs that induce the differentiation of immunosuppressive T_{regs} for the treatment of autoimmune disease (112–116). The regulation of T cell differentiation to immunosuppressive states is already under consideration for the treatment of autoimmune disease (49, 117, 118).

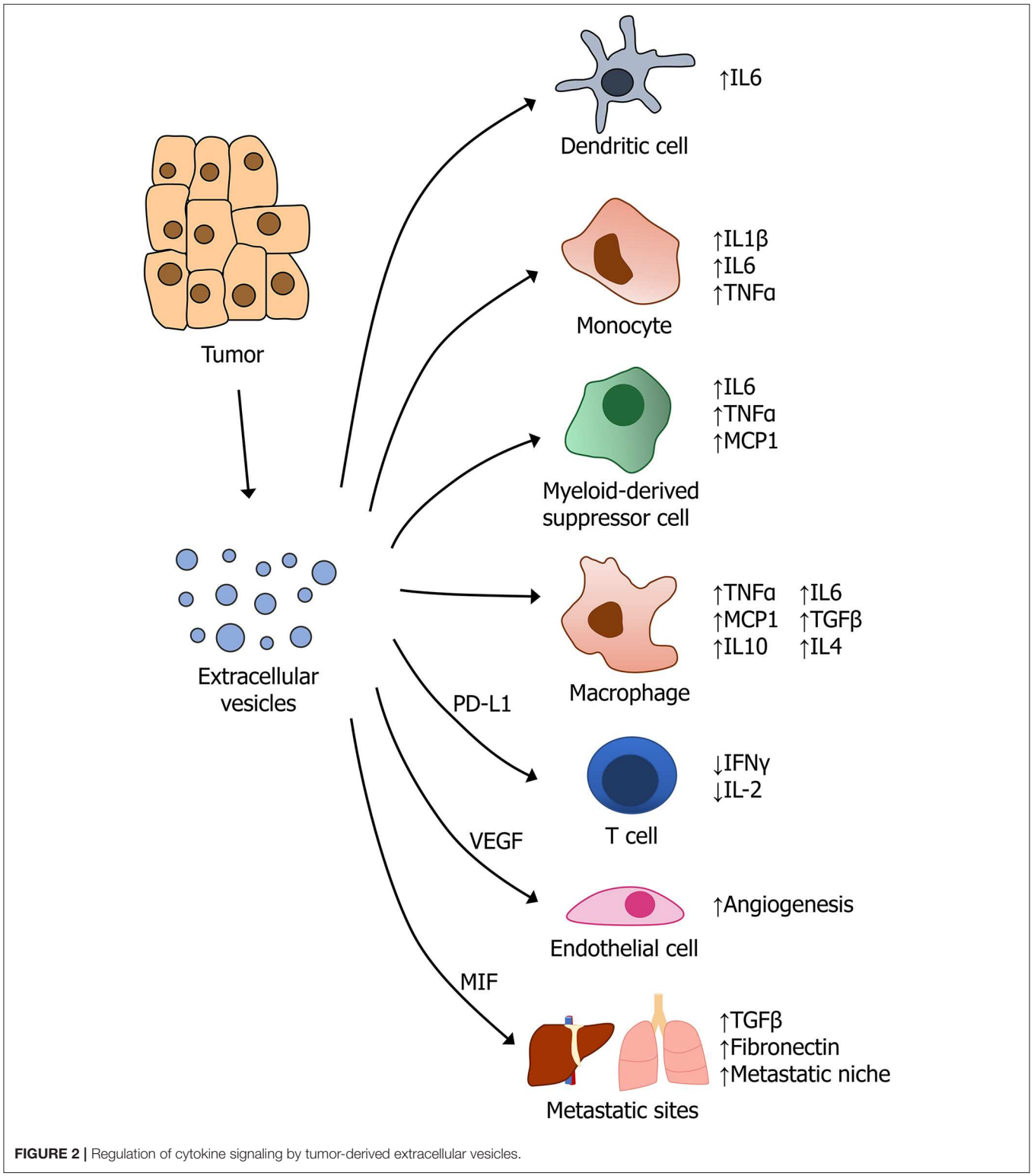

FIGURE 2 | Regulation of cytokine signaling by tumor-derived extracellular vesicles.

Last, determination of the molecular machinery required for EV-associated cytokine secretion, such as ESCRT-dependent or -independent and autophagy-dependent, will provide critical information on select treatments that may target specific pathways [recently reviewed in (119)].

ENGINEERING EVS TO THERAPEUTICALLY DELIVER CYTOKINES

EV encapsulation of cytokines may facilitate their delivery and targeting to distant cells (34). Recent work has demonstrated the

feasibility of engineering EVs to take up proteins as cargo (120–122), presenting a number of techniques by which EVs could be artificially generated to carry cytokine payloads to distant sites. An advantage to this method is that it does not require *a priori* knowledge of the biogenesis pathway resulting in EV cargo loading and secretion. Alternatively, EVs may be targeted to specific cells via binding of EV surface cytokines to cells that express the specific cytokine receptor (123). Sialic-acid binding immunoglobulin lectins, C-type lectins, lactadherin, MHC I, and II receptors, transferrin receptors, tetraspanins, and viral proteins have all been identified as molecules that may promote EV targeting (124–129). Thus, enrichment of exosomes on the basis of their surface ligand expression or ligand enrichment on engineered EVs may be used to induce or inhibit signaling events in recipient cells or to develop receptor-mediated tissue (and cell) targeting (80). Here, we provide two examples of how EVs can be therapeutically modulated for packaging of specific cytokines that drive an immune response. The first example is treatment of bone marrow-derived mast cells with IL4 to drive secretion of exosomes that express MHC II, CD86, LFA-1, and ICAM1, resulting in activation of the adaptive immune arm by inducing proliferation of B and T cells *in vitro* and *in vivo* (130). The second example is from engineering tumor cells to overexpress CD40L, resulting in tumor-derived exosomes that overexpress CD40L to promote dendritic cell maturation, resulting in increased T cell proliferation and antitumor activity *in vivo* (131).

While technological advances in isolating, characterizing, and now engineering EVs to deliver therapeutic payloads and immune modulators are being made [recently reviewed in (80)], it is not until the biological mechanisms by which cytokines are selectively packaged into EVs and the molecular machinery required for secretion determined, that we will be able to fully harness the potential of this natural, physiologic mechanism for cytokine modulation in the context of disease.

AUTHOR CONTRIBUTIONS

BB and CS planned and prepared the manuscript.

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Adipokines and Inflammation Alter the Interaction Between Rheumatoid Arthritis Synovial Fibroblasts and Endothelial Cells

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Objective: The long-distance migration of rheumatoid arthritis synovial fibroblasts (RASFs) in the severe combined immunodeficiency (SCID) mouse model of rheumatoid arthritis (RA) suggests that an interaction between RASFs and endothelial cells (EC) is critical in this process. Our objective was to assess whether immunomodulatory factors such as adipokines and antirheumatic drugs affect the adhesion of RASFs to ECs or the expression of surface molecules.

Methods: Primary ECs or human umbilical vein endothelial cell (HUVEC) and primary RASFs were stimulated with adiponectin (10 μg/mL), visfatin (100 ng/mL), and resistin (20 ng/mL) or treated with methotrexate (1.5 and 1,000 μM) and the glucocorticoids prednisolone (1 μM) and dexamethasone (1 μM), respectively. The expression of adhesion molecules was analyzed by real-time polymerase chain reaction. The interaction of both cell types was analyzed under static (cell-to-cell binding assay) and dynamic conditions (flow-adhesion assay).

Results: Under static conditions, adipokines increased mostly binding of RASFs to EC (adiponectin: 40%, visfatin: 28%, tumor necrosis factor α: 49%). Under flow conditions, visfatin increased RASF adhesion to HUVEC (e.g., 0.5 dyn/cm^2: 75.2%). Reduced adhesion of RASFs to E-selectin was observed after treatment with dexamethasone (e.g., 0.9 dyn/cm^2: −40%). In ECs, tumor necrosis factor α (TNF-α) increased expression of intercellular adhesion molecule 1 (20-fold) and vascular cell adhesion molecule 1 (77-fold), whereas P-selectin was downregulated after stimulation with TNF-α (−6-fold).

Conclusion: The adhesion of RASFs to EC was increased by visfatin under static and flow conditions, whereas glucocorticoids were able to decrease adhesion to E-selectin. The process of migration and adhesion of RASFs to ECs could be enhanced by adipokines via adhesion molecules and seems to be targeted by therapeutic intervention with glucocorticoids.

Keywords: adipokines, endocrine, fibroblast, rheumatoid arthritis, inflammation, endothelium

KEY MESSAGES

- Rheumatoid arthritis synovial fibroblast interacts with endothelial cells under static and flow conditions.
- Adipokines, particularly visfatin, might contribute to RA pathogenesis by increasing RASF adhesion to ECs.
- The therapeutic effect of glucocorticoids in RA may partially be explained by reduced RASF/EC adhesion.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic polyarticular disease, which is characterized by inflammation and joint destruction (1). The RA synovial membrane (synovium), consisting of a lining and sublining layer, is hyperplastic and characterized by increased vascularity and infiltration of immune and stroma cells (1, 2). Rheumatoid arthritis synovial fibroblasts (RASFs) are effector cells and contribute joint inflammation (3, 4). Synovial fibroblasts are able to migrate long distances via the vasculature as previously shown in the severe combined immunodeficiency (SCID) mouse model of RA (4–6), which is mediated by the interaction between RASFs and endothelial cells (ECs) (5). Adhesion molecules and their ligands are involved in the process of migration, which is well-known for immune cell transmigration through vessel walls. Endothelial cells and RASFs are activated by inflammatory factors leading to expression and activation of adhesion molecules, for example, cell adhesion molecules (CAMs) including integrins (7, 8). Upregulation of several adhesion molecules, which mediate adhesion to extracellular matrix (ECM) or cell-to-cell adhesion, is observed in the inflamed RA synovium. For instance, cadherin-11, integrins, and other CAMs are known to be upregulated at sites of inflammation and matrix destruction (7, 9, 10). Cell-to-cell adhesion depends on different adhesion molecules such as selectins that mediate the first steps of adhesion between circulating cells and the endothelium (10). P-selectin and E-selectin are expressed by the endothelium, specifically ECs. Their ligands, such as the E-selectin ligand CD44, Sialyl–Lewis^X, are expressed by circulating cells. Recently, the role of the cell-cell-adhesion molecule E-selectin during EC and RASF interaction has been shown (11). Osteoarthritis synovial fibroblasts (OASFs) showed lower adhesion properties (11). After the first adhesion steps, further CAMs (10), for example, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), are activated and able to interact with other adhesion molecules such as integrins (10).

Both cell-to-cell adhesion and cell-to-ECM adhesion play an important role in inflamed tissues including different compartments of inflamed joints. Of interest, adipose tissue has been found to play a role in inflammatory processes as well (12). Bioactive factors secreted by adipocytes, so-called adipokines (13), have recently been shown to mediate and modulate different inflammatory processes (14). Adipokines induce the secretion of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), complement and growth factors, and the upregulation of different adhesion molecules (15, 16). Both RASFs and ECs are affected by adipokines in RA such as adiponectin, visfatin, and resistin (15, 17, 18).

In obesity, diabetes, atherosclerosis, and metabolic syndrome, altered systemic adiponectin levels have been described (19). In RA, increased adiponectin levels were found to be linked with inflammation (20). However, adiponectin seems to have different effects in different diseases. In metabolic and cardiovascular diseases, antidiabetic and antiatherogenic properties were described for adiponectin (21), whereas in RA, high serum adiponectin levels were associated with radiographic damage (22). Adiponectin stimulates the secretion of IL-8, IL-6, matrix metalloprotease 1 (MMP-1), and MMP-13 by RASFs, which contributes to inflammation and joint destruction (23, 24).

Visfatin and resistin are also upregulated in inflammatory processes including RA (15, 18, 25), and serum levels correlate with disease activity (18, 26). Inhibition of visfatin in a mouse model led to reduced arthritis activity (27). Resistin induces the secretion of, for example, TNF-α, IL-6, IL-12, or IL-1β in different cell types (15) including RASFs (14), and intra-articular injection of resistin has been shown to induce synovitis (17).

These findings as well as several other recent reports suggest that adipokines play an important role in inflammation, as well as matrix remodeling and joint damage in RA (15, 28–30). However, the influence of adipokines on the interaction of RASFs and ECs remains unknown.

Glucocorticoids (GCs) are used in rheumatic conditions since decades (31). The treatment of GCs even reduces radiological progression in RA (32). Glucocorticoids bind to a GC receptor (GR), which is localized in the cytoplasm of cells (33) and consists of distinct domains, that is, a binding domain and domains that interact with DNA (33). If GR is activated by binding GCs, GR-GC is able to move to the nucleus and bind to DNA (33–35). Glucocorticoids increase the synthesis of several anti-inflammatory proteins that can suppress inflammation, that is, lipocortin 1 and IL-1 receptor antagonist, which inhibits the proinflammatory effect of phospholipase A2 and IL-1 (33). The transcription of several proinflammatory cytokines is reduced by GCs, including IL-1β, IL-6, and TNF-α (33). The expression of adhesion molecules can be reduced by GCs (33); that is, the expression of adhesion molecules such as ICAM-1 and E-selectin is inhibited at the level of gene transcription (36).

Therefore, in our study, we evaluated the role of selected adipokines (adiponectin, visfatin, resistin) and GCs (prednisolone, dexamethasone) in RASFs–ECs interactions, particularly with regard to adhesion molecules.

METHODS

Real-Time Polymerase Chain Reaction

RNA was isolated using the RNeasy^{TM} miniprep kit and reverse transcribed (AMV reverse transcriptase; Promega, Walldorf, Germany) using random hexamer primers (Roche Applied Science, Mannheim, Germany). Primer pair (**Supplement 1**) efficiency was tested using the standard curve method considering 2.00 ± 0.05 as acceptable for experiments. Real-time polymerase chain reaction (PCR) was performed using a LightCycler (Roche Applied Science) with SYBR Green I (Roche

Applied Science) as the detection system. Melting curve analysis was used to confirm the specificity of amplification. 18sRNA served as a reference gene. Results were analyzed using the LightCycler software.

Tissues, Cells, and Cell Culture

Bone fragments, cartilage, and synovium from 14 RA patients (**Supplement 7**) were obtained during knee replacement surgeries (Department of Orthopedics and Trauma Surgery, Agaplesion Markus-Hospital, Frankfurt, Germany). Patients met the 1987 American College of Rheumatology classification criteria of RA (37). The study was approved by the local ethics committee of the Justus-Liebig-University Giessen. All patients gave written informed consent. Rheumatoid arthritis synovial fibroblasts were isolated and cultured (maximum seven passages) as described (38). After three passages, supplemented Dulbecco modified eagle medium (DMEM) [20% fetal calf serum (FCS), 1 U/mL penicillin/streptomycin, 1 mM HEPES] was replaced by supplemented RPMI (20% FCS, 1 U/mL penicillin/streptomycin, 1 mM HEPES), and RASFs were cultured at 37°C/5% CO_2 for flow assays. Endothelial cells were isolated from human varicose veins (Departments of Vascular and Cardiac Surgery, Kerckhoff-Klinik, Bad Nauheim, Germany). The vessels were washed twice with phosphate-buffered saline (PBS), and the lumen filled with collagenase H. Ligated vessels were incubated for 1 h at 37°C. Endothelial cell–containing suspension was harvested from the vascular lumen and mixed 1:4 with supplemented DMEM. Cells were centrifuged and resuspended in supplemented DMEM with 0.1 mg/mL EC growth supplement (BD Biosciences, Heidelberg, Germany) and transferred to rat-tail collagen-coated wells. On the next day, adherent cells were washed, and medium changed every 2–3 days for up to three passages to avoid EC dedifferentiation at 37°C/10% CO_2. Solely EC cultures without fibroblast contamination (vimentin/CD31 immunocytochemical confirmation) were used. At 100% confluence, cells were detached and placed in rat-tail collagen-coated plates. Human umbilical vein endothelial cells (HUVECs) (Promocell, Heidelberg, Germany) were cultured on coated plates with supplemented DMEM with 0.1 mg/mL EC growth supplement for up to two passages.

Cell-to-Cell Binding Assay

Rheumatoid arthritis synovial fibroblasts were cultured in 12 well-plates and stimulated for 17 h with adiponectin (10 µg/mL; BioVendor, Brno, Czech Republic), visfatin (100 ng/mL; BioVendor), resistin (20 ng/mL; Peprotech, Hamburg, Germany), TNF-α (10 ng/mL; R&D, Bio-Techne Germany, Wiesbaden-Nordenstadt, Germany), prednisolone (1.0 µM; Mibe GmbH, Brehna, Germany), dexamethasone (1.0 µM; Mibe GmbH), or methotrexate (MTX, 1.5 or 1,000 µM; Medac GmbH, Wedel, Germany). The stimulation with TNF-α was used as a positive control as its proinflammatory role, and its ability to increase adhesion molecules in RASFs and ECs is well-known (39–41). The concentrations used for stimulation were based on dose–response analyses with visfatin (25) and adiponectin (42) as performed by our group for previous publications. The concentration for resistin was based on the level that could be detected in synovial fluid (43). The concentration for resistin was based on the level that could be detected in synovial fluid (43). The concentrations of dexamethasone and prednisolone were selected according to publications using both types of GCs to repress inflammation in RASFs (44, 45). The lower MTX concentration corresponds to serum levels as found in RA therapy [MTX (RA)] (46, 47), whereas the higher dose corresponds to serum levels of cancer therapy [MTX (C)] (48, 49). After 17 h, cells were washed with PBS. The stimulation time was chosen based on preliminary experiments that demonstrated an optimal response of stimulation with adipokines after 17 h (data not shown). Viability of RASFs was confirmed by calcein-AM staining for 30 min. Cells were detached with Accutase (Thermo Fisher Scientific GmbH, Dreieich, Germany), and 5×10^3 cells were added to confluent EC layers pretreated for 17 h with TNF-α (R&D) and incubated together for 1 h at 37°C. Supernatants were removed, replaced with serum-free medium (RPMI), and then shaken for 5 min at full speed of the orbital shaker (300 rpm) to remove loosely attached RASFs. This washing step was repeated three times in total using serum-free medium. The medium was removed using a suction device. Attached fluorescent RASFs (on unstained ECs) were quantified in five representative areas each. Confluence of the EC layer was confirmed using bright field microscopy. Results were compared to unstimulated RASFs.

Flow-Adhesion Assay

Capillary slides (µ-Slide $VI^{0.4}$, ibiTreat-pretreated; Ibidi, Gräfelfing, Germany) were coated with 30 µL recombinant human E-selectin (1 mg/mL E-selectin/Fc-chimera, 1:20 in PBS) for 1 h at room temperature. Human umbilical vein endothelial cells were added into capillary slides and grown to 100% confluence overnight. Human umbilical vein endothelial cells were activated for 17 h with TNF-α (10 ng/mL; R&D) in DMEM with 0.1 mg/mL EC growth supplement. Rheumatoid arthritis synovial fibroblasts 1.1×10^6 were cultured in supplemented RPMI and stimulated for 17 h with adiponectin, visfatin, or resistin, as well as prednisolone, dexamethasone, or MTX. The stimulation time was chosen based on preliminary experiments that demonstrated an optimal response of stimulation with adipokines after 17 h (data not shown). Results were compared to nonstimulated RASFs. After washing with PBS, RASFs were detached with Accutase, and 0.8×10^6 RASFs were transferred into a syringe pump (Model-100-Series; KD Scientific, Holliston, MA, USA) and connected to the capillary slide. Synovial fibroblast migration through capillaries was monitored microscopically. Cells slowly rolling over the surface and arrested cells were quantified (**Figure 1**). Means of rolling/arresting cells per visual field were calculated for each recorded sequence (3×1 min each). Flow rates of 18.4, 30.5, or 60.5 mL/h, respectively, correspond to shear stress of 0.5, 0.9, or 1.8 dyn/cm^2, respectively, representing rates detected in postcapillary venules (50, 51).

Statistics

Data in figures are shown in percentages as box–whisker plots with median, 25th/75th percentile (box), and lowest/highest value (whisker) using SPSS Statistics 24 (IBM, Armonk, New York, United States of America).

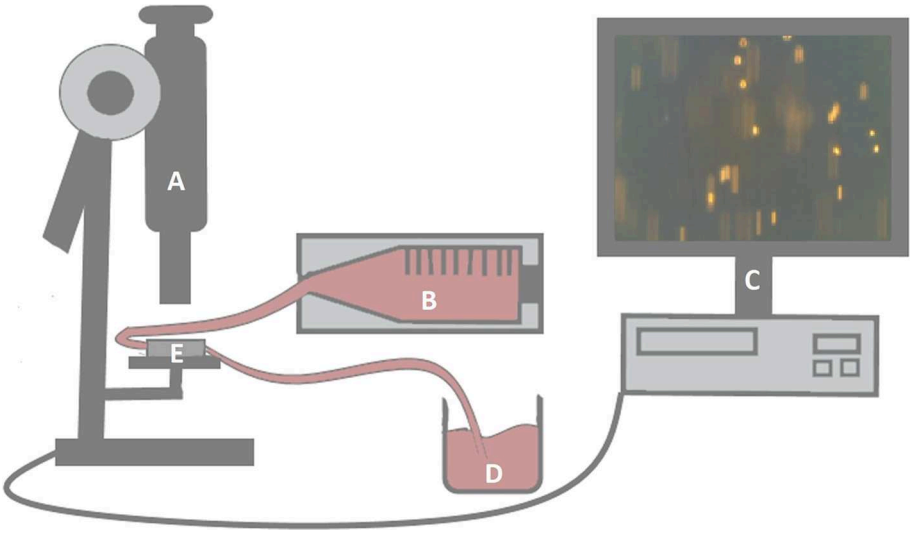

FIGURE 1 | Experimental setup of the flow-adhesion assay. Capillaries **(E)** were monitored microscopically **(A)**. Flow rates of RASF-containing suspensions were regulated by a syringe pump **(B)**. The pump was connected to the capillaries by a tube. Another tube was connected to a collection vessel **(D)** after passing through the capillaries. Synovial fibroblast migration was evaluated by three video sequences per setting **(C)**.

In order to analyze adipokine-mediated alteration at different treatments linear mixed models were applied to analyze the repeated measurement design using SPSS Statistics 24 (IBM). Data were log or log2 transformed to reach normal distribution of the residuals, which was verified by Q-Q plots. Estimated marginal means (rhombus) for the fitted models were described together with 95% confidence intervals (CIs). Treatment differences were described by estimated difference and their 95% CIs. All multiple comparisons were Bonferroni adjusted within the analysis of each outcome.

Means, differences, and boundaries of CI were anti–log transformed for the presentation of the results. Issues were regarded as significant for $p \leq 0.05$. Fold changes of the RT-PCR data were regarded as significant if the 95% CI of log2 transformed $-\Delta\Delta$ct values did not contain 0.

RESULTS

Effects of Adipokines and GCs on Adhesion Molecule Gene Expression by RASFs

First, we investigated the influence of adipokines and GCs on the gene expression of selected adhesion molecules. Stimulation with TNF-α increased expression of VCAM-1 (**Figure 2A**, 16.4-fold, 95% CI = 4.9–55) and ICAM-1 (**Figure 2B**, 20.3-fold, 95% CI = 6.1–68) significantly. Dexamethasone (**Figure 2A**, −5.1-fold, 95% CI = 0.095–0.408) and prednisolone (**Figure 2A**, −3.2-fold, 95% CI = 0.136–0.717) downregulated expression of VCAM-1 significantly. In contrast, none of the adipokines, GCs or MTX, affected the expression of the integrin subunits α2, α4, αv, β1, and β5 on RASFs (data not shown). Expression of cadherin-11 (data not shown), which is overexpressed in RA-synovium (52), as well as VCAM-1 (**Figure 2A**) and ICAM-1 (**Figure 2B**), was not significantly changed after stimulation with adipokines or antirheumatic drugs.

Influence of Adipokines and GCs on Gene Expression of EC Adhesion Molecules

Tumor necrosis factor α stimulation resulted in a significant overexpression of VCAM-1 (**Figure 3A**, 77-fold, 95% CI = 11.8–499, **Supplement 3**) and ICAM-1 (**Figure 3B**, 20.3-fold, 95% CI = 6.1–68, **Supplement 3**). Expressions of VCAM-1 and ICAM-1 were not affected by adipokines and antirheumatic drugs (**Figures 3A,B**), but most of the cell samples showed a decrease of expression of ICAM-1 after stimulation with adiponectin (**Figure 3B**). Expression of P-selectin was not changed by adipokines and antirheumatic drugs (**Figure 3C**), whereas stimulation with TNF-α significantly decreased expression (−6.3-fold, 95% CI = 0.069–0.37, **Supplement 3**).

RASF Adhesion to ECs Under Static Conditions

Cell-to-cell binding of RASFs to confluent EC layers was increased after stimulation with selected adipokines (adiponectin: 40%, visfatin: 28%, resistin: 30%) compared to nonstimulated control, which was set to 0% (**Figure 4**). The results for visfatin ($p = 0.03$, **Supplement 4**) and adiponectin ($p = 0.048$, **Supplement 4**) were significant. Tumor necrosis factor α, as proinflammatory cytokine, led to a significantly increased adhesion (49%; $p = 0.004$, **Supplement 4**). Treatment with dexamethasone did not alter adhesion (**Figure 4**). Although adhesion of both cell types was not changed significantly, most of the cell samples showed a decrease of adhesion in response to prednisolone (8/10) and MTX (C and RA, 4/6 each; **Figure 4**).

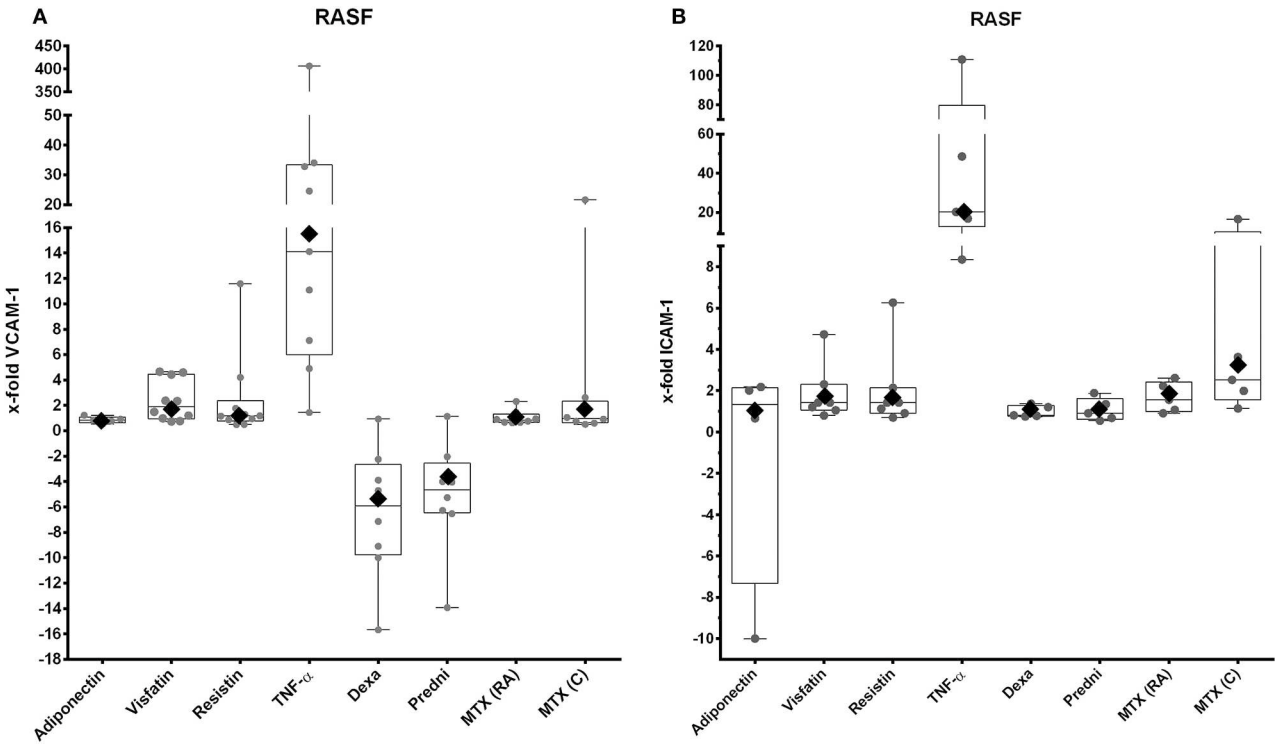

FIGURE 2 | VCAM-1 and ICAM-1 expression by RASFs after stimulation with selected adipokines and therapeutics. Results were compared to non-stimulated controls. **(A)** mRNA expression of VCAM-1 by RASFs after stimulation with adiponectin ($n = 5$), visfatin ($n = 10$), resistin ($n = 10$), TNF-α ($n = 9$), or therapeutics ($n = 8$ each). Adipokines and methotrexate did not have any effect on expression of VCAM-1, whereas TNF-α upregulated expression of VCAM-1 [16.4-fold (rhombus), 95% CI = 4.9–55], but not significantly. Dexamethasone (−5.09-fold, 95% CI = 0.095–0.408) and prednisolone [−3.2-fold (rhombus), 95% CI = 0.14–0.717] downregulated expression of VCAM-1. **(B)** mRNA expression of ICAM-1 by RASFs after stimulation with adiponectin ($n = 4$), visfatin ($n = 7$), resistin ($n = 7$), TNF-α ($n = 5$), and therapeutics (each $n = 5$). Adipokines and therapeutics did not have any effect on expression of ICAM-1. Tumor necrosis factor α significantly upregulated expression of ICAM-1 [20.4-fold (rhombus), 95% CI = 6.07–68].

RASF Adhesion to E-Selectin and HUVECs Under Flow Conditions

Rheumatoid arthritis synovial fibroblast attachment to E-selectin (**Figures 5A,B**) and HUVECs (**Figures 5C,D**) was evaluated in flow-chamber assays. Unstimulated RASFs showed rolling and/or adherence to E-selectin and HUVECs in all settings as shown previously (53). Stimulation with visfatin led to an increased adhesion of RASFs to E-selectin [18.4 mL/h: 16.3%, 30.5 mL/h: 35.7%, 60.5 mL/h: 27.4%; **Figure 5A**, not statistically significant (NS)]. Resistin (18.4 mL/h: −0.9%, 30.5 mL/h: 6%, 60.5 mL/h: 17%) and TNF-α (18.4 mL/h: 15.4%, 30.5 mL/h: 35.9%, 60.5 mL/h: −17.7%) did not significantly change RASF adhesion to ECs. Treatment with dexamethasone (**Figure 5B**) reduced interaction of RASFs with E-selectin significantly (8.4 mL/h: −40.9%, 30.5 mL/h: −40%, 60.5 mL/h: −29.7%, **Supplement 5**). Although prednisolone (18.4 mL/h: −36.9%, 30.5 mL/h: −26.3%, 60.5 mL/h: −26.6%) and MTX (RA) (18.4 mL/h: −33.7%, 30.5 mL/h: −4.5%, 60.5 mL/h: −15.1%) reduced adhesion of RASFs to E-selectin in most patients, the results were not statistically significant (**Supplement 5**). Methotrexate (C) had no effect on the binding of RASFs to E-selectin.

During cell migration, not only E-selectin is involved in cell interaction and adhesion. Therefore, capillaries were coated with TNF-α-activated HUVECs. Stimulation of RASFs with visfatin (**Figure 5C**) resulted in significantly ($p = 0.002$, **Supplement 6**) increased adhesion of RASFs to HUVECs (18.4 mL/h: 75.2%, 30.5 mL/h: 37.9%, 60.5 mL/h: 49.8%). Similar results were observed after stimulation with TNF-α, particularly at lower flow rates (18.4 mL/h: 64.2%, 30.5 mL/h: 24.6%, 60.5 mL/h: 20%, NS). Resistin did not change RASF adhesion significantly (18.4 mL/h: 37.4%, 30.5 mL/h: −0.8%, 60.5 mL/h: 0.8%), although the lowest flow rate was elevated in nearly all samples evaluated. Dexamethasone (18.4 mL/h: −3.6%, 30.5 mL/h: −20.9%, 60.5 mL/h: −22.7%) and prednisolone (18.4 mL/h: −21.4%, 30.5 mL/h: −43.1%, 60.5m l/h: −30.2%) did not change adhesion of both cell types significantly (**Figure 5D**, **Supplement 6**), but most of the cell samples showed decreased adhesion.

Methotrexate application (RA and C) increased variation in RASF adhesion in all settings (**Figure 5D**).

DISCUSSION

Rheumatoid arthritis synovial fibroblasts play a crucial role in joint damage (38) due to their ability to invade and degrade cartilage and bone and to migrate through the vasculature to distant joints (5). We evaluated in comparison

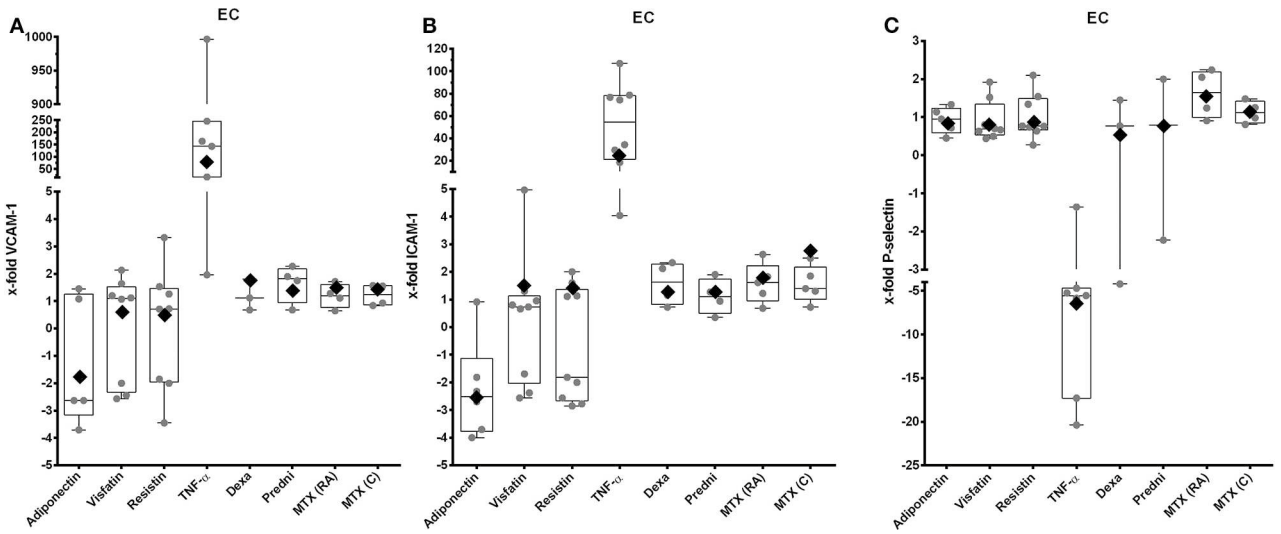

FIGURE 3 | VCAM-1, ICAM-1, and P-selectin expression by EC after stimulation with selected adipokines and therapeutics. Results were compared to non-stimulated controls. **(A)** mRNA expression of VCAM-1 by primary EC after stimulation with adiponectin ($n = 5$), visfatin ($n = 10$), resistin ($n = 10$), TNF-α ($n = 9$), or therapeutics ($n = 8$ each). Adipokines and therapeutics did not have any effect on expression of VCAM-1, whereas TNF-α significantly upregulated expression of VCAM-1 [76.8-fold (rhombus), 95% CI = 11.8–499]. **(B)** mRNA expression of ICAM-1 by primary EC after stimulation with adiponectin ($n = 6$), visfatin ($n = 9$), resistin ($n = 9$), TNF-α ($n = 8$), glucocorticoids ($n = 4$ each), or methotrexate ($n = 5$ each). Adiponectin led to a significant increased expression of ICAM-1 [−2.5-fold (rhombus), 95% CI = 0.26–0.6]. Resistin visfatin and therapeutics did not have any effect on expression of ICAM-1, whereas TNF-α significantly upregulated expression of ICAM-1 [37.02-fold (rhombus), 95% CI = 15–91.4]. **(C)** mRNA expression of P-selectin by primary EC after stimulation with adiponectin ($n = 5$), visfatin ($n = 8$), resistin ($n = 8$), TNF-α ($n = 7$), glucocorticoids (each $n = 3$), or methotrexate (each $n = 4$). Adipokines and therapeutics did not have any effect on expression of P-selectin. Tumor necrosis factor α significantly downregulated expression of P-selectin [−6.3-fold (rhombus), 95% CI = 0.069–0.37].

to therapeutic modulation of inflammation whether adipokines have an influence on the interaction between RASFs and ECs by modulating the expression of adhesion molecules on the respective cell surfaces.

In RA, increased angiogenesis takes place because of an imbalance of proangiogenic and anti-angiogenic factors (54). Proinflammatory factors activate the endothelium leading to upregulation of adhesion molecules. Because of these inflammatory processes, hemodynamics is altered, leading to reduced bloodstream velocity. This allows cells, such as lymphocytes and also RASFs, to interact with adhesion molecules on activated ECs. Tumor necrosis factor α induces the expression of adhesion molecules on ECs (55), and TNF-α serum levels are increased in RA (56).

P-selectin is one of the pivotal adhesion molecules in this process. It is located in Weibel–Palade bodies in ECs and translocated to the cell surface following stimulation (57). Recent data suggest an active role of P-selectin in RA (58), and soluble P-selectin is known to be elevated in the serum of RA patients and to correlate with disease activity (58). In our study, stimulation of ECs with selected adipokines did not change the expression of P-selectin in ECs, whereas TNF-α downregulated its expression significantly. Recent data show that P-selectin reaches the maximum of protein expression after 2 h of stimulation with TNF-α (59). After 2 h, a time-dependent downregulation of mRNA and protein expression could be observed (59), which is in line with the observed P-selectin reduction after 17 h in our study. Additionally, flow conditions are required to activate the conformation of P-selectin. Adhesion to P-selectin stops if there is no flow (51), for example, after vessel occlusion.

Similar to the leukocyte adhesion cascade, RASFs were able to interact with ECs via E-selectin under flow conditions, representing one of the first binding partners for leukocytes (5, 10). Similar to P-selectin, flow conditions are required to activate E-selectin (60). Below 18.4 mL/h (0.5 dyn/cm²), selectins do not change to their activated conformation. A flow rate of 60.5 mL/h (1.8 dyn/cm²) or higher leads to a concentration of cells in the center of the vessel or capillary, and no interaction with the endothelium is possible (50).

In our flow-adhesion assay using E-selectin–coated capillaries, addition of dexamethasone resulted in a significant decrease of RASF adhesion. Thus, dexamethasone might diminish interactions between E-selectin and its ligands, for example, Sialyl–Lewis^X (53), as well as CD44, which is also expressed by RASFs (61, 62). Glucocorticoids inhibit the nuclear factor κB pathway (63), potentially influencing adhesion between cells and ECM. In contrast to RASFs, OASFs showed a reduced rolling/adhesion capability to E-selectin in previous experiments (11).

Primary venous ECs and HUVECs showed comparable findings regarding cell numbers in previous experiments (11). Because of the limited availability of ECs, we performed the flow-adhesion assay with HUVECs. In HUVEC-coated capillaries, stimulation with visfatin resulted in a significantly increased adhesion of RASFs to TNF-α-activated ECs. This might be due to induced expression of adhesion molecules on

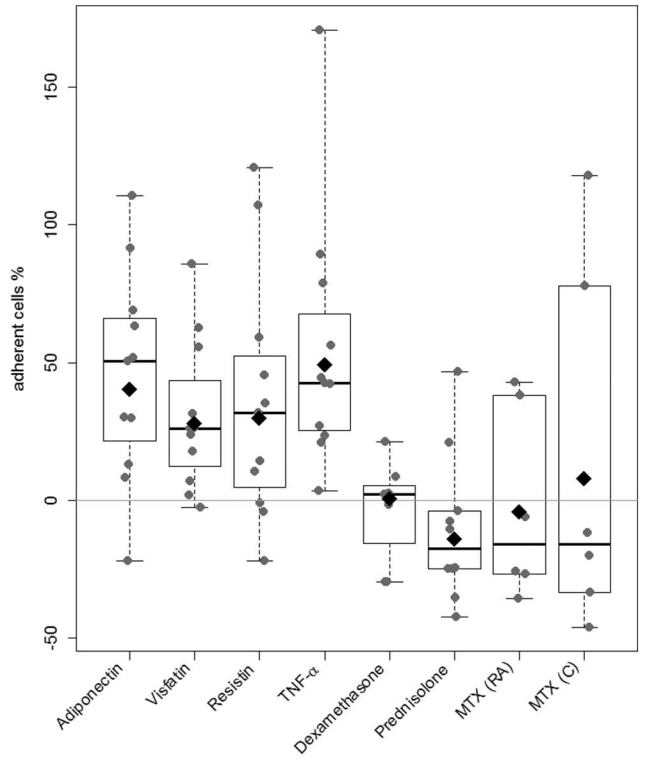

FIGURE 4 | Evaluation of RASFs binding to EC. A cell-to-cell binding assay using primary EC was used to evaluate the effect of the adipokines adiponectin, visfatin, resistin, and TNF-α ($n = 8$ each), glucocorticoids (prednisolone, dexamethasone, $n = 7$ each), and methotrexate ($n = 6$ each dosage). Unstimulated RASFs served as control. The percentage of change in adherent RASFs compared to unstimulated RASFs was calculated. Data in figure are shown in percentages as dot plots with estimated marginal means. Stimulation with selected adipokines increased and prednisolone decreased adhesion to EC in most of the cell samples (adiponectin: 26%, visfatin 19%, resistin 17%, prednisolone: −15%, NS).

RASFs by visfatin (64). Stimulation with resistin did not show comparable effects in E-selectin- or HUVEC-coated capillaries, although adhesion to HUVECs was increased at the lowest flow rate.

The binding to selectins is followed by the interaction between CAMs and integrins (10). Several integrins of circulating cells, for example, on leukocytes or RASFs, bind to CAMs of ECs, for example, VCAM-1 and ICAM-1. Stimulation of RASFs with adipokines as well as antirheumatic drugs did not change the expression of selected integrins (integrin α2, α4, αν, β1, and β5, data not shown). In contrast, stimulation of ECs with adiponectin reduced ICAM-1. In the context of cardiovascular diseases, adiponectin showed protective effects (21). This could be related to a downregulation of adhesion molecules on ECs. However, because of the chronic inflammatory environment within the synovial tissue, this effect may not be sufficiently strong to reduce influx of cells from the bloodstream into the inflamed joints.

Soluble forms of VCAM-1 and ICAM-1 could be detected in higher concentration in serum of RA patients (65), and both molecules are increased on different cells of the hyperplastic RA synovium (66), including RASFs (41, 67). Ligands of VCAM-1 and ICAM-1 are expressed by leukocytes allowing interactions of both cell types (67, 68). Stimulation of RASFs with TNF-α upregulated the expression of both adhesion molecules significantly. After treatment with dexamethasone and prednisolone, a significant decrease of VCAM-1 mRNA expression was observed. The reduced expression of VCAM-1 by RASFs may diminish adhesion of RASFs to other cells and decrease RASF activity in RA.

The increased adhesion of RASFs to ECs compared to OASF under static conditions was confirmed in cell-to-cell binding assays in previous experiments (11). Under static conditions, stimulation with adiponectin and visfatin increased adhesion of RASFs to ECs significantly, which was comparable to the effect of TNF-α especially after stimulation with adiponectin. Because of the absence of flow conditions, selectins are not involved in cell–cell interactions in this assay. However, the increased RASF–EC binding might be due to the proinflammatory effect of adipokines in RA (69) as expression of the measured adhesion molecules was not altered. Increased adhesion is likely to be promoted by other factors, for example, activation of other adhesion molecules (integrins) and rearrangement on the cell surface (70) besides induced expression of the selected, as well as other adhesion molecules. Stimulation with prednisolone decreased adhesion of RASFs to ECs in most of the cell samples, but results did not reach statistical significance. The decrease of adhesion could be mediated directly or indirectly by altered gene transcription due to GCs. The expression of VCAM-1 was decreased significantly after stimulation with dexamethasone and prednisolone, which may lead to a reduced cell–cell interaction (**Figure 4** and **Supplement 2**).

In RA, RASFs and ECs are located within an inflammatory environment, which contributes to the activation of RASFs and ECs (10, 71). Secreted chemokines and cytokines lead to activation of integrins on the cell surface, as well as induction and activation of other adhesion molecules (10, 51). Our data support the idea that adipokines might play a role in immunomodulation in RA. Especially visfatin enhanced the interaction of RASFs with ECs under flow conditions. *Vice versa*, corticosteroids were able to downregulate VCAM-1 expression in ECs and to reduce adhesion of RASFs to E-selectin under flow conditions. This could explain why corticosteroids are successful in slowing down RA progression. The identification of target molecules responsible for increasing cell adhesion could therefore open new opportunities for RA therapy by targeting these molecules to slow RA progression.

CONCLUSION

In this project, we could show that certain adipokines lead to an increase in the adhesion of RASFs to ECs under static and dynamic conditions.

This result suggests that distinct adipokines promote the adhesion of RASFs to the endothelium and thus primarily promote the initial steps of the disease process in the context of the adhesion cascade. The use of dexamethasone and prednisolone resulted in a reduction of RASF adhesion to

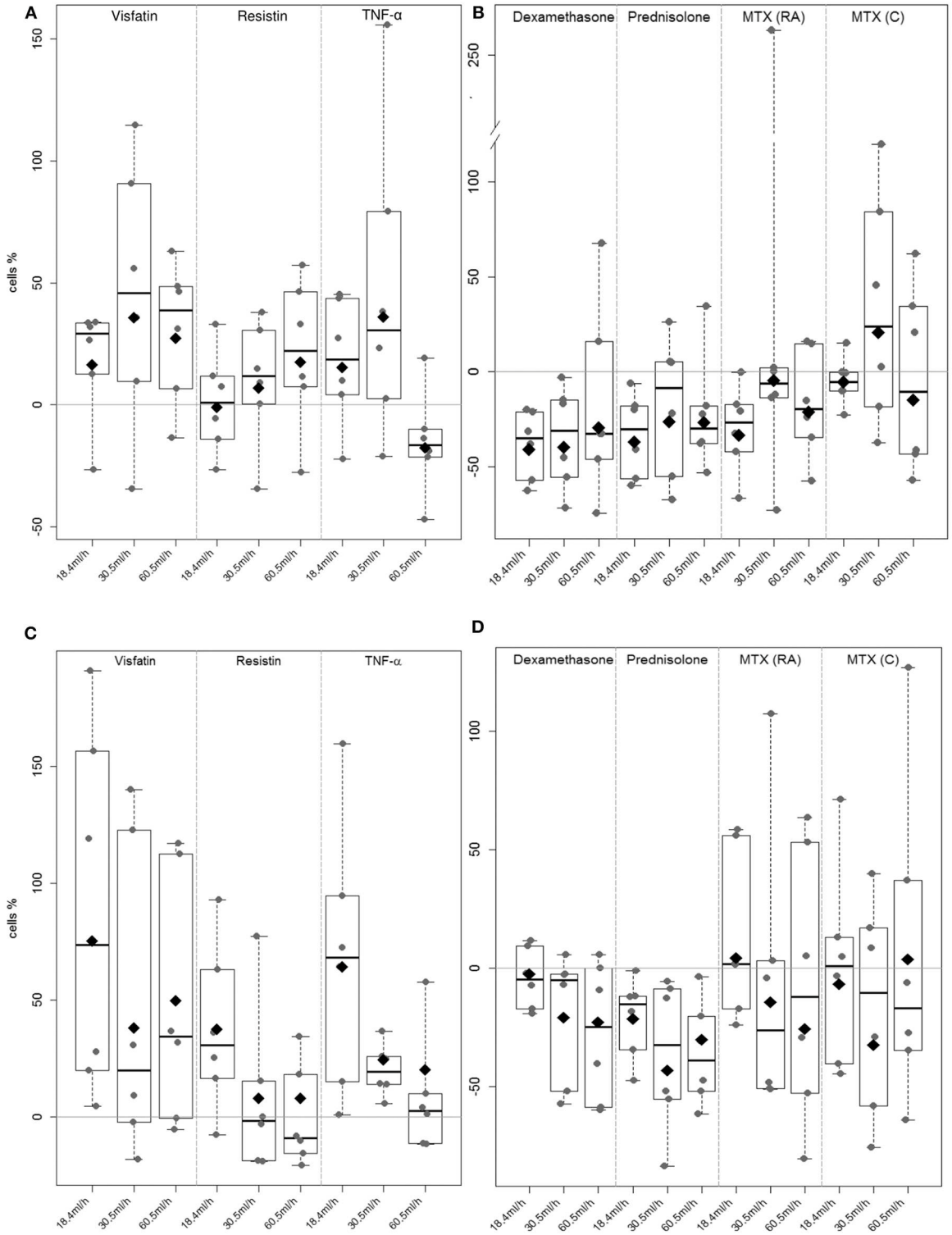

FIGURE 5 | Rheumatoid arthritis synovial fibroblast adhesion to E-selectin and HUVEC under flow conditions. A flow adhesion assay was used to evaluate the effect of the selected adipokines visfatin, resistin, TNF-α (A & C) and therapeutics prednisolone, dexamethasone and methotrexate (B & D) to E-selectin and HUVEC (each $n = 6$). Unstimulated RASF served as control. The percentage of change in adherent RASF compared to unstimulated RASF was calculated. **(A)** Visfatin increased adhesion to E-Selectin in most of the samples (NS). **(B)** Stimulation with dexamethasone significantly ($p = 0.043$) decreased adhesion to E-selectin coated capillaries (8.4 ml/h: −40.9%, 30.5 ml/h: −40%, 60.5 ml/h: −29.7%). **(C)** Significant increase ($p = 0.002$) of adhesion to HUVEC could be observed after stimulation with visfatin (18.4 ml/h: 75.2%, 30.5 ml/h: 37.9%, 60.5 ml/h: 49.8%). **(D)** Stimulation with therapeutics did not reach any significant change in adhesion.

ECs, especially under flow conditions. This might provide an additional explanation for the protective effect of GCs, which are used in RA therapy.

Interestingly, stimulation with GCs even reduced expression of VCAM-1 by the RASFs, which could affect the binding of leukocytes. This could reduce the recruitment of leukocytes, which could lead to a lower number of immune cells that are maintained in the synovium and contribute to the disease process.

Taken together, the results might open new therapeutic opportunities as, for example, the effect of adipokines could be selectively blocked by antibodies. In addition, the anti-inflammatory effect of TNF-α blockers or the basic drugs (e.g., MTX) could be amplified by adipokine blockers. In contrast, the blockage of a single proinflammatory adipokines is most likely not sufficient to achieve a complete remission of RA, but is worth to consider the combination of the blockade of proinflammatory adipokines and antirheumatic drugs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Justus-Liebig-University Giessen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EN and UM-L designed experiments. RH performed the research, analyzed and interpreted the data, and wrote the manuscript. Synovial fibroblasts from patients with rheumatoid arthritis came from department of orthopedics and trauma surgery, Agaplesion Markus Hospital Frankfurt (SR), and EC came from department of cardiac surgery, Kerckhoff-Klinik Bad Nauheim (MSchö). MSchw, M-LH, CS, MA, and MD contributed to preparation of the research. JP-K contributed to analyze and interpret the data. IT and UL edited the manuscript before submission. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00925/full#supplementary-material

Supplement 1 | Primer sequences for VCAM-1, ICAM-1, and P-Selectin.

Supplement 2 | Evaluation of gene expression after stimulation in RASF. Dexamethasone and prednisolone down-regulated expression of VCAM-1 which was significant. ICAM-1 and VCAM-1 expression was up-regulated after stimulation with TNF-α significantly.

Supplement 3 | Evaluation of gene expression after stimulation in EC. TNF-α induced expression of VCAM-1 and ICAM-1 significantly. Adiponectin lead to a significant increased expression of ICAM-1. P-Selectin was significantly down-regulated after stimulation with TNF-α for 17 h.

Supplement 4 | Cell-to-Cell binding assay. Stimulation with adiponectin, visfatin, and TNF-α significantly increased adhesion to EC.

Supplement 5 | Flow adhesion assay with E-Selectin-coated channels for all velocities. Stimulation with dexamethasone significantly decreased adhesion to E-selectin for all velocities.

Supplement 6 | Flow adhesion assay with HUVEC-coated channels for all velocities. Stimulation with visfatin significantly increased adhesion to HUVEC for all velocities.

Supplement 7 | Patient characteristics.

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Potential T Cell-Intrinsic Regulatory Roles for IRF5 via Cytokine Modulation in T Helper Subset Differentiation and Function

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Interferon Regulatory Factor 5 (IRF5) is one of nine members of the IRF family of transcription factors. Although initially discovered as a key regulator of the type I interferon and pro-inflammatory cytokine arm of the innate immune response, IRF5 has now been found to also mediate pathways involved in cell growth and differentiation, apoptosis, metabolic homeostasis and tumor suppression. Hyperactivation of IRF5 has been implicated in numerous autoimmune diseases, chief among them systemic lupus erythematosus (SLE). SLE is a heterogeneous autoimmune disease in which patients often share similar characteristics in terms of autoantibody production and strong genetic risk factors, yet also possess unique disease signatures. *IRF5* pathogenic alleles contribute one of the strongest risk factors for SLE disease development. Multiple models of murine lupus have shown that loss of *Irf5* is protective against disease development. In an attempt to elucidate the regulatory role(s) of IRF5 in driving SLE pathogenesis, labs have begun to examine the function of IRF5 in several immune cell types, including B cells, macrophages, and dendritic cells. A somewhat untouched area of research on IRF5 is in T cells, even though *Irf5* knockout mice were reported to have skewing of T cell subsets from T helper 1 (Th1) and T helper 17 (Th17) toward T helper 2 (Th2), indicating a potential role for IRF5 in T cell regulation. However, most studies attributed this T cell phenotype in *Irf5* knockout mice to dysregulation of antigen presenting cell function rather than an intrinsic role for IRF5 in T cells. In this review, we offer a different interpretation of the literature. The role of IRF5 in T cells, specifically its control of T cell effector polarization and the resultant T cell-mediated cytokine production, has yet to be elucidated. A strong understanding of the regulatory role(s) of this key transcription factor in T cells is necessary for us to grasp the full picture of the complex pathogenesis of autoimmune diseases like SLE.

Keywords: autoimmune disease, Th1, Th2, Th17, polarization

INTRODUCTION

T cells are responsible for balancing a variety of regulatory and effector functions. Many of these roles are accomplished through the expression of a panel of cytokines controlled by a specific cohort of transcription factors. These cytokines can act to initiate, support or inhibit different T cell effector functions and, during homeostatic conditions, maintain a tight immunological balance between pro- and anti-inflammatory T cell functions. In the case of immune-mediated diseases, the balance between T cell subsets is often disrupted. For instance, patients with systemic lupus erythematosus (SLE) demonstrate an increase in T helper 1 (Th1) relative to T helper 2 (Th2) cells and a dysregulated balance between Th1 and T helper 17 (Th17) cells, while results from single cell sequencing of patients with rheumatoid arthritis (RA) have demonstrated a skewing toward Th1 effector memory CD4+ T cells, and a murine model of multiple sclerosis (MS) showed resistance to disease development due, in part, to a loss of key T cell intrinsic Th1 mediators (1–4). In SLE studies performed in humans and mice, some of the likely cytokine inflammatory mediators and immunomodulatory agents identified as participating in disease development include (but are not limited to) interferon (IFN)-α, IFN-γ, tumor necrosis factor (TNF), interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, IL-21 and transforming growth factor (TGF)-β (5–8). Although many of these cytokines are produced by various antigen presenting cells (APCs) to help initiate effector T cell responses, all of these cytokines are also produced in varying quantities by the effector T cells themselves. Sustained T cell response, both appropriate and pathogenic following the initial priming event, depends greatly on the ability of the T cells to both produce the appropriate cytokines and reformat their transcriptional landscape at an epigenetic level to generate a positive feedback loop. Dysregulation of this positive feedback loop or inappropriate epigenetic reprogramming could result in a T cell-driven dysregulation of pro- or anti-inflammatory cytokine production, as seen in numerous autoimmune diseases (9–11). This review will delve specifically into the potential roles for IRF5 in the regulation of effector T cell decision and maintenance with a focus on Th1, Th2 and Th17 subsets, whose high interconnectivity has been demonstrated to be impacted by IRF5 deletion or hyperactivation. However, continued research into a potential role(s) for IRF5 in the other T cell subsets, particularly follicular helper T (Tfh) cells and regulatory T (Treg) cells, is an important next step in the elucidation of autoimmune disease pathogenesis via IRF5 dysregulation. For a more general review on the role of cytokines in autoimmune disease, see Raphael et al. (12).

Th1 Cells

Th1 effector cells regulate the body's defense against viruses, bacteria and intracellular pathogens and, when properly functional, are vital members of the immunological homeostasis required to maintain our health. However, dysregulation of Th1 cells has been implicated as a key player in the global immunological dysfunction that results in many autoimmune disease conditions, among them RA, SLE, MS, type 1 diabetes mellitus, idiopathic thrombocytopenic purpura, and experimental allergic encephalomyelitis (1, 2, 13–19). Th1 cells are traditionally defined by their production of IL-2 and IFN-γ and by expression of the transcription factor and epigenetic modifier, T-bet, a member of the T-box family of transcription factors (20–22). In the subsequent decades following the initial characterization of these defining factors, critical roles for the DNA-binding regulatory proteins signal transducer and activator of transcription 4 (STAT4), STAT1, and STAT5 in the development and support of Th1 subsets have also been revealed (23, 24). Briefly, naïve CD4+ T cells are stimulated to develop into Th1 effector cells by IL-12 binding to the IL-12 receptor (23, 25). Once activated, Th1 cells produce IL-2 and IFN-y. IL-2 acts as a potent inducer of both T cell proliferation and T cell effector fate decision (26). IFN-y employs both stimulatory and inhibitory roles to maintain Th1 effector dominance. IFN-y can induce the phosphorylation of STAT1, thereby increasing expression of the Th1 specific genes, IL-12 receptor beta 1 (*IL12RB*) and T-box transcription factor 21 (*TBX21*; encoding T-bet). Increased levels of T-bet results in positive feedback on T-bet expression through T-bet activation of *IFNG* transcription. T-bet also increases STAT1 activation and mediates the upregulation of Th1-specific genes including *IL12R*, which will in turn signal to increase STAT4 phosphorylation and dimerization. STAT4 itself can act as a potent transcriptional repressor of genes that would normally support Th2 differentiation (i.e., *GATA3*) and acts in concert with T-bet to promote the positive feedback loop resulting in increased IFN-γ production.

This feedback loop enhancing Th1 differentiation also has built in inhibitory mechanisms. T-bet can bind to and inhibit BCL-6 (B-cell lymphoma 6 protein) early in Th1 polarization, preventing transcription of genes involved in alternative effector fates (27). T-bet and BCL-6 comprise two lineage-defining factors that cooperate in the regulation of Th1 gene expression patterns (28). However, later in Th1 activation T-bet recruits BCL-6 to the *IFNG* promoter, resulting in inhibition of *IFNG* transcription and thereby shutting down one of the main drivers of the Th1 effector response (23, 28, 29). In addition, T-bet increases the transcription of the membrane protein T cell immunoglobulin mucin-3 (Tim-3) in later stages of Th1 differentiation, which acts as an inhibitor of the Th1 response upon binding to the ligand, β-galactosidase-binding lectin 9 (Gal-9) (30, 31). Gal-9 regulates Th-induced proinflammatory cytokine production (32). Further supporting the concept that dysregulation of T-bet can result in a pathologically imbalanced immune system, Tim-3 blockade has been shown to result in autoimmune disease development (33). Interestingly, most of T-bet's transcriptional regulatory capabilities have been shown to occur through epigenetic modifications of genetic loci using either H3K4 (activating) or H3K27me3 (inactivating) chromatin methylation patterns. In fact, production of the key Th1 driving cytokine IFN-γ is dependent on both chromatin remodeling by T-bet and increased IL-12R expression through direct T-bet transcriptional activity (29, 34–36). However, much less has been published with regards to the direct negative regulation of

T-bet activity in activated Th1 cells and how dysregulation at the level of T-bet could result in rampant Th1 activation and the development of autoimmune disease.

As previously described, T-bet clearly plays an indispensable role in the positive feedback loop governing Th1 effector subset polarization. T-bet both positively regulates ∼50% of Th1-specific genes and inhibits Th2-specific gene transduction, including GATA3, the Th2-specific transcription factor (29). Interestingly, ∼70% of Th2-specific genes in Th1 cells are still bound by GATA3. In this scenario, GATA3 is bound by T-bet and inhibited from transducing Th2-specific transcripts in Th1 effector cells (37, 38). Other sources show that T-bet can also directly interact with and recruit GATA3 away from its Th2 gene loci. In either case, it is hypothesized that part of the rationale for skewing toward a Th2 phenotype upon loss of negative regulation by *TBX21* is due to both increased *GATA3* transcription and increased GATA3 association with Th2-specific genetic loci (29).

A Conserved DEF6-IRF5-T-bet Regulatory Axis Mediates Th1 Effector Response Through T-bet

Th1 cells are capable of producing the cytokines granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, TNF-β, and IFN-γ (39). As previously described, uncontrolled positive feedback of these cytokines on T cells can result in an imbalance between T cell subsets and their secreted cytokines, resulting in the development of autoimmune disease pathologies (40). Here we will explore the role of IRF5 in regulating an appropriate Th1 immune response and how loss of IRF5 may cause effector T cell dysregulation.

In the full-body *Irf5* knockout (KO) mouse, the majority of studies have shown that there is skewing of T cells toward a Th2 effector phenotype with an accompanying decrease in Th1 effector subsets, thereby implicating a role for IRF5 in Th1 effector T cell commitment and/or maintenance (41–44). However, the T cell intrinsic IRF5-dependent molecular and genetic systems at play in these regulatory mechanisms governing Th1 feedforward and inhibitory loops have yet to be thoroughly explored. Based on previously published work, it seems likely that a main target for the dysregulation of Th1 effector T cells resulting in a substantial decrease in Th1 effector fate decision and a concomitant increase in Th2 cells would involve dysregulation of the master transcriptional regulator, T-bet. However, IRF5 does not play a role in the direct transcriptional regulation of this key transcription factor (45). This does not preclude the possibility that IRF5 interacts with T-bet on a protein level. In the following paragraphs, we propose a novel DEF6-IRF5-T-bet regulatory mechanism that controls Th1 effector T cell polarization.

The SWEF family of Rho-GTPase regulatory proteins consists of two family members, switching B cell complex subunit (SWAP70) and DEF6 (also known as IRF4 binding protein, IBP, or SWAP70-like adaptor of T cells, SLAT) (46, 47). Recent publications have shown that SWAP70 and DEF6 (also recently identified as a potential risk variant in human SLE) bind to and sequester IRF5 in the nucleus of age-associated B cells (ABCs) (46, 48). In naïve CD4+ T cells, the predominant SWEF family member expressed is DEF6 (47). As in ABCs, the importance of DEF6 as a master regulator has become increasingly evident through continuing discoveries of its roles in many aspects of T cell regulation, including IRF4 modulation, cytoskeletal kinetics and protein expression control through mammalian target of rapamycin complex 1 (mTORC1) regulation (49–53). In addition, as also observed in ABCs, upon T cell stimulation IRF5 levels are shown to dramatically increase (43, 54). If a similar pattern is followed in T cells as in ABCs, elevated levels of IRF5 may allow it to escape inhibition by DEF6 and perform its crucial regulatory role(s) in T cells (**Figure 1**).

So what regulatory role(s) might IRF5 play in Th1 cells? Upon stimulation of ABCs with IFN-γ and IL-21, levels of IRF5 increase, thereby allowing IRF5 to escape its negative regulation by SWEF family members, translocate to ABC transcriptional sites, and recruit T-bet to ABC-specific T-bet binding motifs (46, 55). It would be interesting to examine if this similar chromatin remodeling by T-bet followed by transcriptional activation resulting specifically from IRF5 driven T-bet recruitment in ABCs is a conserved mechanism for epigenetic and transcriptional regulation in T cells. In this scenario, IRF5 deletion would also likely result in GATA3 release from T-bet, allowing increased GATA3 translocation and binding to Th2-specific cytokine promoters, resulting in increased Th2-specific genes and cytokines (**Figure 1**). A mechanism similar to this one has already been alluded to in the $Irf5^{-/-}$ pristane-induced model of lupus, where loss of *Irf5* results in an increase in the production of the Th2-specific cytokines IL-4 and IL-5 (41, 42, 56).

The proposed inclusion of IRF5 in the regulation of T-bet through a conserved interaction with DEF6 in T cells will likely have direct implications in our understanding of the control of cytokine release by T cells and the T cell-driven pathogenesis of several autoimmune diseases. An example of this is regulation of the runt-related transcription factor 3 (*RUNX3*) gene. RUNX3 enhances IFN-γ production and inhibits IL-4 production when recruited to the promoter regions by its interaction with T-bet or other members of the T-bet family. Upon Th1 activation, RUNX3 has been shown to be transcriptionally activated by T-bet. RUNX3 will then form a complex with T-bet and translocate to the promoters of IFN-γ and IL-4, activating and inhibiting their transcription, respectively, to maintain the Th1 positive feedback loop. Our hypothesis is that T-bet recruitment to sites of transcriptional regulation is mediated by IRF5 (**Figure 1**). In a 2016 paper examining effects of *Runx3* polymorphisms, they identified an IRF4 binding site upstream of the *Runx3* promoter (57). In 2019, this same group identified that this area in the promoter region could also mediate binding of other transcription factors, including IRF5 (57). Loss of *Runx3* compromises IFN-γ production and abrogates inhibition of IL-4, thereby implying a vital role for RUNX3 in maintaining effector T cell polarization (58, 59).

Many key regulatory signaling and transcriptional proteins are expressed in both B and T cells. If IRF5 is indeed required to recruit T-bet to its transcriptional loci, loss of *IRF5* would result in decreased efficiency of T-bet initiation of its Th1

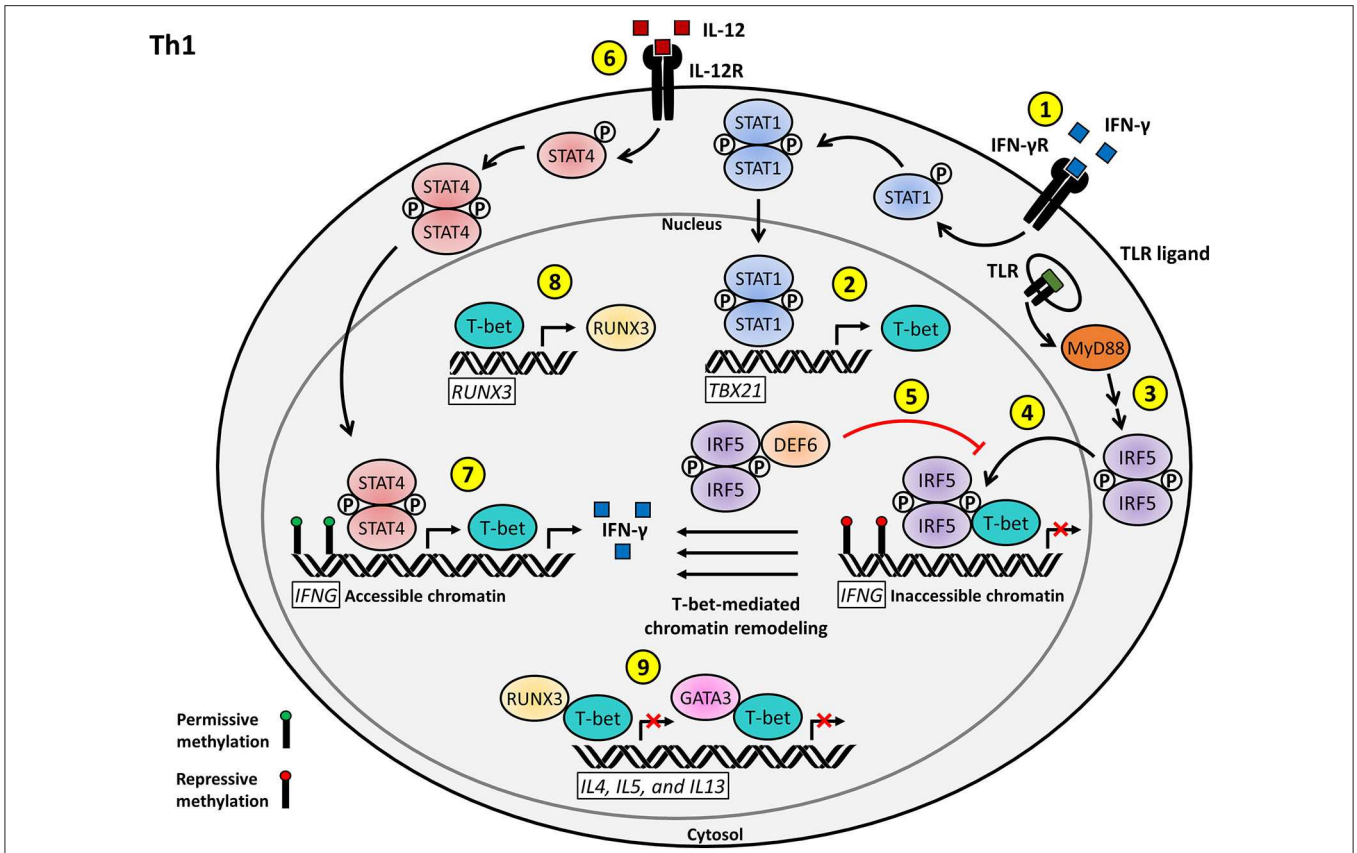

FIGURE 1 | Proposed model for the T cell-intrinsic role of IRF5 as a positive regulator of Th1 effector function and differentiation. (1) IFN-γ stimulation of the IFN-γR on naïve CD4+ T cells induces STAT1 activation and nuclear translocation. (2) Phosphorylated STAT1 activates the transcription of *TBX21*, leading to the production of T-bet. (3) T cell stimulation, possibly through TLR signaling, induces IRF5 activation and nuclear translocation. (4) Nuclear IRF5 recruits T-bet to the silenced *IFNG* locus to facilitate permissive T-bet-mediated chromatin remodeling. (5) DEF6 binds to nuclear IRF5 in order to inhibit IRF5-mediated T-bet recruitment to the *IFNG* locus. (6) IL-12 signaling through the IL-12R results in STAT4 activation and nuclear translocation. (7) Phosphorylated STAT4 and T-bet induce the transcription of the accessible *IFNG* locus and subsequent IFN-γ signaling drives Th1 effector differentiation. (8) T-bet also acts as a positive regulator of *RUNX3* transcription. (9) T-bet interacts with RUNX3 and GATA3 to inhibit the transcription of Th2 signature genes, including *IL4, IL5,* and *IL13*, to promote Th1 polarization.

transcriptional program, leading to a defect in Th1 effector subset polarization, as seen in *Irf5* KO models. In addition, dysregulation of DEF6 could directly impact regulation of the key Th1 transcriptional driver, T-bet, providing a mechanism by which *DEF6* polymorphisms contribute to SLE risk (**Figure 1**).

Th2 Cells

Th2 effector T cells are involved in the defense against parasitic infections, allergic reactions and the resolution of chronic inflammation (60). Unlike the previously discussed Th1 effector cells, the mechanisms driving Th2 differentiation are still not fully understood. Dendritic cells are thought to play a distinct role in supporting Th2 effector decision. However, they are incapable of producing the key Th2 mediating cytokine, IL-4 (61). Interestingly, IL-4 produced by CD4+ T cells themselves has been shown to be sufficient in initiating the Th2 response (62). These findings support the hypothesis that Th2 effector decision may be a default response in conditions where there is a lack of stimuli driving other Th effector fates. High levels of GATA3 expression in naïve CD4+ T cells prime the cells for Th2 differentiation, providing additional evidence for this theory. GATA3 is only downregulated upon initiation of T effector cell polarization into alternative subsets (61, 63).

Upon initiation of Th2 polarization, the principle Th2 cytokine, IL-4, acts in a stimulatory capacity through activation of STAT6 phosphorylation. Phosphorylated STAT6 increases the transcription of *IL4* and *GATA3*. GATA3 is both a vital component of the machinery required for *IL4*, *IL5,* and *IL13* transcription and is required for the global epigenetic remodeling needed to achieve Th2 polarization (64). The importance of GATA3 in Th2 effector differentiation is demonstrated by the consequences resulting from loss of *GATA3*. Even in the absence of the key Th1 cytokines, IFN-γ and IL-12, lack of *GATA3* drives Th1 polarization (65).

Increased chromatin accessibility mediated by GATA3 both leads to the secretion of the Th2 specific cytokines, IL-4, IL-5, and IL-13, and inhibits the production of the Th1 specific cytokine, IFN-γ. Interestingly, IRF4 has recently been shown to act as an additional positive regulator of *IL4* transcription during Th2 differentiation (49, 66). IRF4 forms a complex with

GATA3 and the chromatin organizer special AT-rich binding protein 1 (SATB1) in order to bind to the RHS6 sequence during Th2 differentiation, located ubiquitously throughout the Th2 cytokine locus. All three of these factors are required in order for Th2-specific genes to be expressed (67). GATA3 has also been proposed to act in a positive feedback loop through the induction of IRF4 (68). The emerging roles of the complex transcriptional and regulatory networks involving the master transcription factor and epigenetic modulator, GATA3, are still being explored.

As in Th1 cells, there are regulatory mechanisms in place to inhibit the transcription of alternative T effector subset mediators upon Th2 effector commitment. One of these factors is the ubiquitous regulator, Ikaros (a hemopoietic-specific zinc finger protein also known as IKZF1). Regulatory functions for Ikaros have been implicated in almost all T helper cell subsets and loss of *Ikaros* has been shown to be detrimental in the maintenance of Th2 commitment. In $Ikaros^{null}$ CD4+ T cells there is general hypoacetylation of the Th2 cytokine locus, increased IFN-γ production in Th2 polarizing conditions, decreased production of IL-4, IL-5, and IL-13, decreased GATA3 and c-MAF expression, and increased levels of T-bet and STAT1. All of these factors result in a skewing from Th2 to Th1 (69). Despite this growing pool of knowledge on the regulatory mechanisms ascribed to Ikaros, very little is known about the regulation of Ikaros itself in T cells (60, 70). However, a recent study of Ikaros regulation in B cells may provide insight into a conserved IRF5-dependent Ikaros regulatory mechanism in T cells (45).

A Conserved MyD88/IRF4/IRF5 and Ikaros Regulatory Mechanism Mediates the Th1-Th2 Balance

As previously discussed, the regulation of Th2 cells, and thus the closely related Th1 effector subset, is still not fully understood. Pathologic skewing toward a Th2 response has been shown to result in atopic disorders, such as systemic sclerosis, and immunosuppression through the dysregulated production of their hallmark cytokines, IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (40). IL-4, IL-10, and IL-4-induced IL-10 production in particular, has an inhibitory role on Th1 effector cells, thereby further contributing to the skewing from a Th1 to a Th2 phenotype and mediation of Th1 effector response (71, 72). Here we will explore a potential role for IRF5 in the control of Th2 subsets and how dysregulation can contribute to an enhanced pathogenic Th1 effector response.

A key role for IRF4 in Th2 subset development and, more specifically, the control of IL-4 production, has previously been identified (73). Although the precise regulatory mechanisms at play for IRF4 in T cells have yet to be fully elucidated, levels of IRF4 have been shown to be higher in resting Th2 cells compared to Th1 and Th17 (49). Inquiries into the role of IRF5 in other immune cell types have revealed alternative roles for IRF4 outside that of direct transcriptional regulation. In macrophages, IRF4 has been established as a negative regulator of Toll-like receptor (TLR) signaling by directly competing with IRF5 for binding to myeloid differentiation primary response gene 88 (MyD88) (74). MyD88 acts as a scaffold protein where IRF5 can receive its post-translational modifications from its interacting modulators. MyD88 functions downstream of all TLRs except TLR3 (75). Inhibition of the IRF5-MyD88 interaction by IRF4 results in attenuation of inflammatory cytokine production downstream of TLR signaling (48, 74, 76). However, the impact of T cell TLR signaling on intrinsic CD4+ T cell effector function and the pathological conditions that result from dysregulation are still by and large unconfirmed (77, 78).

The main body of research on the impacts that dysfunction of TLR signaling at the level of MyD88 might have on T cells examined how loss of *MyD88* in upstream signaling cells (macrophages and dendritic cells) impacted Th2 differentiation. Little has been done to examine the specific roles and pathways of TLR signaling in T cells (75, 79). Mounting evidence implicates the TLR/MyD88 pathway as a potential regulatory mechanism in the Th1/Th2 effector decision. A study performed using the *B. burgdorferi* model of infection in a T cell-specific *MyD88* deletion model demonstrated that loss of *MyD88* in T cells results in an intrinsic defect in the Th1 and Th17 response. Th2 effector response was unfortunately not examined (80). However, an OVA-based murine $MyD88^{-/-}$ model of asthma showed significant defects in Th2 effector response upon stimulation (81). Taking these findings into account, we postulate that MyD88 plays an intrinsic role in T effector cell differentiation alongside IRF4. While IRF4 is expressed at high levels in Th2 effector cells, low levels of IRF5 are associated with a Th2 response. In Epstein-Barr Virus (EBV)-infected B cells, IRF4 was shown to be a negative transcriptional regulator of *IRF5* (82). If *IRF5* is no longer transcribed at high enough levels to initiate a transcriptional response tailoring an alternative T cell fate through TLR signaling, a Th2 transcriptional profile maintained by IRF4 through the previously described mechanisms can be maintained. This theory is supported by the T cell-specific *MyD88* KO. Here, removal of another key player in the IRF5 TLR signaling pathway results in Th2 skewing, akin to the results seen in the *Irf5* KO (80). Along with the conserved expression of IRF4 and IRF5 and the as of yet undefined mechanism by which IL-4 is initially regulated, we postulate that a conserved IRF4/IRF5/MyD88 axis in effector T cells may be playing a role in IRF5 activation and the skewing between Th1 and Th2 subsets (**Figure 2**).

Ikaros is another mediator that plays an important role in the maintenance of Th2 effector subset decision. Ikaros is a hematopoietic transcription factor that directly associates with Th2 regulatory gene loci and is involved in the positive regulation of Th2 gene expression (69). Ikaros has a binding site in its promoter region for IRF4, IRF5, and IRF8. In B cells, IRF8 and IRF5 both bind and regulate the *Ikzf1* promoter, IRF8 acts in an activating capacity while IRF5 acts as an inhibitor. Inhibition of *Ikzf1* transcription by IRF5 allows for the assumption of B cell antibody class switching to IgG2a/2c (45). Expression and function of these IRF transcription factors and Ikaros are conserved in T cells. If IRF5 were to maintain a similar negative regulatory function for Ikaros as seen in B cells, loss of *IRF5* would allow unimpeded Ikaros activation, resulting in

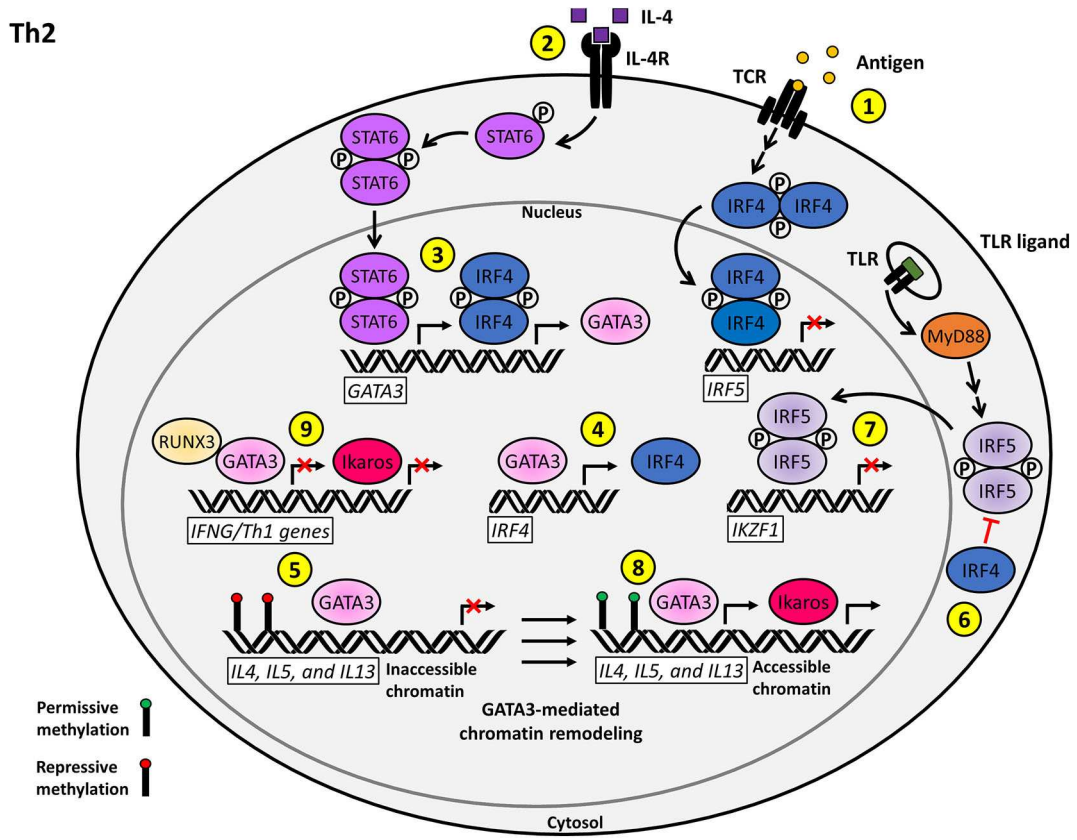

FIGURE 2 | Proposed model for the T cell-intrinsic role of IRF5 as a negative regulator of Th2 effector function and differentiation. (1) Antigenic stimulation of the TCR induces the upregulation of IRF4, which acts as a repressor of *IRF5* transcription. (2) Stimulation of the IL-4R by IL-4 on naïve CD4+ T cells induces STAT6 activation and nuclear translocation. (3) Phosphorylated STAT6 synergizes with IRF4 to activate *GATA3* transcription. (4) A positive regulatory loop supported by GATA3-mediated *IRF4* transcription augments Th2 polarization. (5) GATA3 induces permissive chromatin remodeling at the *IL4, IL5,* and *IL13* Th2 cytokine locus. (6) IRF4 inhibits the TLR-induced activation of IRF5 via antagonizing the interaction between MyD88 and IRF5. (7) Nuclear IRF5 functions as a negative regulator of *IKZF1* transcription, which limits the production of Ikaros. (8) Ikaros and GATA3 promote the transcription of accessible Th2 polarizing genes including *IL4, IL5,* and *IL13*. (9) Ikaros and GATA3 further reinforce the Th2 phenotype via repression of the Th1 transcriptional network.

a shift toward Th2 effector polarization. On the other hand, overexpression or hyperactivation of IRF5, as seen in SLE patients, could lead to loss of Ikaros transcription and a shift from the Th2 to Th1 effector T cell subset. In support of this theory, Gene Ontology shows that *IKZF1* has distal sites for T-bet and GATA3 binding (37). As previously discussed, we postulate that there is likely a role for IRF5 in the regulation of T-bet through direct interaction, as well as one for GATA3, by extension (**Figure 2**). Thus, there is increasing circumstantial evidence of a regulatory role for IRF5 in the control of Ikaros function, either through direct binding or through the recruitment of chromatin remodeling agents.

Th17 Cells

T cell development is highly dependent on the surrounding cytokine environment and is characterized by high degrees of plasticity which, in many cases, can serve a pathogenic role. This is especially seen in the case of dysregulated Th17 cells, which have been associated with many immunological diseases including RA, inflammatory bowel disease (IBD), SLE, MS, psoriasis and cancer (20, 83, 84). Although Th17 effector subsets have been considered for drug targets to counteract the dysfunctional immune systems that they help to support, our lack of knowledge about the pathways regulating the polarization of these cells toward pathogenic phenotypes has hindered our choice(s) of a specific target (83). Recently, the monoclonal antibody against IL-17R, marketed under the name Brodalumab (AMG827), has entered clinical trials and was shown to be effective in improving psoriasis (85). However, many other drugs on the market attempting to initiate an IL-17 blockade have been met with mixed results depending on the disease setting (86). Thus, although Th17 effector function is strongly implicated as a potential target for future drug development, we need to gain a better understanding of the mechanisms controlling Th17 pathologic phenotypes and how these can drive autoimmune disease.

In SLE patients, it has been shown that hyperactive IRF5 results in skewing toward a Th1 and Th17 phenotype. However, to say simply "Th17 phenotype" is an oversimplification of the

diversity of this particular T cell subset. Th17 effector T cells exist in a gradient between classical and pathogenic which is determined in part by the cytokine milieu they are exposed to. In the pathogenic state, there are two opposing directions that Th17 cells can follow—either toward a Th1-like phenotype, which is often associated with autoimmunity or toward a more Th2-like state, which is correlated with enhanced immunosuppression (83). At steady state, Th17 cells differentiate into Tfh cells and support immunoglobulin A (IgA) production by germinal center B cells. IL-23 in particular, although not required for Th17 differentiation, is required for pathogenic Th17 maintenance and survival (87).

IL-17A, the "pathogenic" cytokine produced by Th17 cells, has been shown to be a key player in the perpetuation of inflammation associated with autoimmune tissue damage. IL-17A functions through several mechanisms including the activation of other immune cells, increasing B cell functions, recruiting neutrophils, Treg mediation and enhancing proinflammatory cytokine release (20, 88, 89). In the mouse model of human MS (murine experimental autoimmune encephalomyelitis, EAE), blocking the interaction between IL-17 and IL-17 receptors resulted in substantial attenuation of EAE development (90). Unfortunately, the picture painted by this interaction is oversimplified. To date, there have been six different IL-17 cytokines identified, IL-17A–F, and five unique versions of the IL-17 receptor, IL-17RA–RE. For a more extensive review on what is known about the functional variations of these family members, see Swaidani et al. (91) and Jin and Dong (92). Although IL-17A has been identified as the main mediator of inflammation associated with autoimmune disease, the pathways downstream of IL-17A binding to IL-17R are still not fully defined.

In non-disease states, Th17 cells serve an important function in supporting tailored immune responses to various pathogens (20). Th17 effector cells maintain a balance between the alternative Treg differentiation pathway and conversion into a Th1-like phenotype. IRF4 has been shown to be a key mediating factor in maintaining the balance between Th17 and Tregs. *Irf4* KO results in an increase in the Treg FoxP3 (forkhead box P3) transcription factor and a decrease in RORγt (RAR-related orphan receptor gamma t), the major transcription factor for commitment to Th17 fate in part through transcriptional upregulation of IL-17 (93). The relatively one-sided conversion from Th17 to Th1 seems to be controlled through stimulation from circulating cytokines. Stimulation of Th17 polarized cells by IL-12 and IFN-γ results in inhibition of IL-17 secretion and conversion to a more Th1-like state, characterized by increased levels of STAT4 and T-bet expression. Increased levels of TGF-β inhibit this plasticity and result in maintenance of a stable Th17 phenotype. Early STAT transcription factors are also at play in the regulation of Th17 decision; STAT3 promotes and STAT5 inhibits Th17 differentiation (23). Because of the plasticity of the Th17 subset and its ability to interconvert between many other effector-like subsets in response to disease, the regulation of this particular subset is complex and still not well-understood. However, it has been established that maintenance of the inflammatory state that characterizes many autoimmune diseases is in part due to the IL-17-initiated positive feedback loop from defective Th17 cells (94, 95).

A Potential IRF5-Mediated T Cell-Intrinsic Feedback Loop Regulates the Th17 Effector Decision Through Inflammatory Cytokine Production, STAT3, Ikaros and IL-10

The role of IRF5 in Th17 effector cells is still an open field. However, based on the previous mechanisms described, especially those relating to Th1 regulation, a role for IRF5 in Th17 differentiation and plasticity seems highly likely. Several studies have supported a role for IRF5 in Th17 effector differentiation, although few, if any, studies have yet to examine an intrinsic role for IRF5 in Th17 cells. Loss of *Irf5* in murine models of severe asthma resulted in decreased IFN-γ and IL-17 responses upon ovalbumin (OVA) immunization (96). In an *Irf5* KO antigen-induced arthritis (AIA) model, Th1, Th17, and γδ IL-17 producing T cells were found to have significantly decreased effector responses following immunization with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant (CFA). In addition, this model showed decreased levels of *Ifng* and *Il17a* mRNA and the key Th1 and Th17 cytokines IL-1β, IL-6, IL-12, and IL-23 (97).

The cytokines that are often used to characterize pro-inflammatory Th17 subsets are IL-22, GM-CSF, and IFN-γ. Interestingly, several of these inflammatory cytokine mediators are also known to induce the expression of IRF5. In macrophages, increased IRF5 expression results in an M1 (inflammatory) macrophage phenotype through the upregulation of IL-12, TNF-α, and IFN-γ, with concomitant repression of IL-10 (98). Through binding to various promoter regions, IRF5 also increases *IL6*, *IL12,* and *IL23p19* transcription. Interestingly, pathogenic Th17 cells also secrete IL-12, IL-23, IL-6, and IFN-γ in addition to various other Th17-specific effector cytokines and transcription factors (66, 99). A conserved role for IRF5 in the transcriptional activation of these inflammatory cytokines in Th17 cells should be explored.

IL-10 production by Th17 cells may provide yet another avenue for a potential role for IRF5 in the regulation of Th17-mediated inflammation (100). Although the regulation of IL-10 in Th17 effector cells is not fully understood, it has been well-established that IL-10 is required for T cells to maintain control over Th17 effector function (101). One of the mechanisms by which IL-10 expression is mediated is through TGF-β and IL-6. These two factors work to activate the c-MAF transcription factor through STAT3, which in turn activates *IL10* transcription by binding to the *IL10* promoter (102). IL-10 acts to reduce IL-17 and IFN-γ production, thus negatively regulating pro-inflammatory Th17 effector reactions. Interestingly, in the context of Newcastle disease virus (NDV)-infected Balb-C mice, IRF5 was shown to induce *Stat3* transcription in the presence of undetectable levels of the cytokines IL-6 and IL-10 (103). In addition, IRF5 has been shown to be a key mediator of *IL6* transcription in human pDCs (104). IRF5 was also shown to be upregulated by the janus kinase 2 (JAK2)/STAT3

pathway in human umbilical vein endothelial cells (105). The existence of a positive feedback loop between STAT3 and IRF5 in Th17 cells, where activation of IRF5 transcription downstream of STAT3 allows for IRF5 to feedback and increase IL-6 and STAT3 expression, should be explored as a potential mechanism by which IRF5 mediates Th17 effector response (**Figure 3**).

In an alternative regulatory arm, Ikaros has been shown to be required for inhibition of heterochromatic remodeling at the gene loci for the Th17 effector program. Ikaros has also been shown to repress expression of both *FOXP3* and *TBX21*, which both normally act to negatively regulate Th17 development (106, 107). However, T-bet was also reported to positively regulate transcription of the *IL23R* by binding to a specific site in the *IL23R* promoter sequence and inducing IFN-γ expression by Th17 cells, thus inducing a pro-inflammatory state (108, 109). Hence, depending on the location and context of T-bet expression in Th17 cells, T-bet can initiate or ameliorate inflammatory responses. The precise mechanisms through which these regulatory actions are achieved have not yet been established. In addition to binding to and regulating *FOXP3* and *TBX21* expression, Ikaros has a binding site specifically within the *IL10* promoter and acts to positively regulate IL-10 production. It is likely that Ikaros has other, as of yet, undefined epigenetic and transcriptional regulatory roles to support Th17 effector functions (106, 110). A hint as to additional regulatory mechanisms involved in the pathways leading to Th17 effector commitment comes through literature on IRF5 regulation. In macrophages, IRF5 has been shown to have both positive and negative effects on *IL10* transcription through direct binding to the *IL10* promoter (98, 111). In Th17 cells, IL-10 plays a crucial role in the downregulation of the pro-inflammatory cytokines, IL-17 and IFN-γ. As loss of *IRF5* results in a decrease in Th17 effector subsets, this could imply a positive regulatory role for IRF5 in a conserved pathway, either through an inhibitory role at the *IL10* promoter (as seen in macrophages), a negative role in Ikaros regulation (as described in B cells) or induction of a STAT3–IRF5 positive feedback loop as previously described (**Figure 3**).

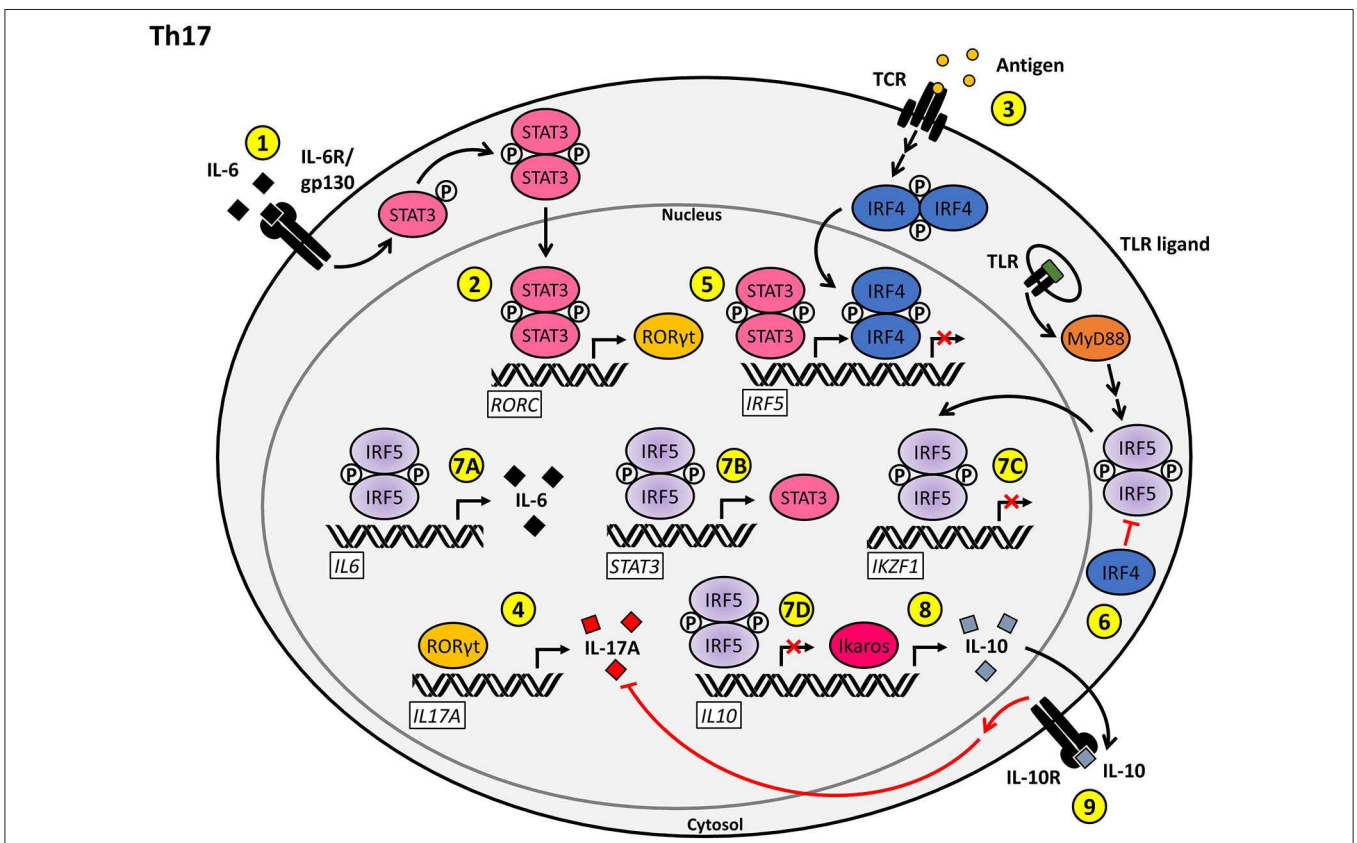

FIGURE 3 | Proposed model for the T cell-intrinsic role of IRF5 as a positive regulator of Th17 effector function and differentiation. (1) Stimulation of the IL-6R/gp130 complex by IL-6 on naïve CD4+ T cells induces STAT3 activation and nuclear translocation. (2) Phosphorylated STAT3 induces the transcription of *RORC,* leading to the production of RORγt. (3) Antigenic stimulation of the TCR induces the upregulation of IRF4. (4) RORγt induces the transcription of *IL17A*, driving Th17-mediated inflammation. (5) The transcription of *IRF5* is positively regulated by phosphorylated STAT3 and negatively regulated by IRF4. (6) IRF4 inhibits the TLR-induced activation of IRF5 via antagonizing the interaction between MyD88 and IRF5. (7A–D) Nuclear IRF5 promotes the Th17 phenotype through upregulation of *IL6* (7A) and *STAT3* (7B) expression and repression of *IKZF1* (7C) and *IL10* (7D) transcription. (8) Ikaros functions as a positive regulator of *IL10* transcription. (9) IL-10 signaling through the IL-10R antagonizes the production of IL-17A, thereby inhibiting Th17 inflammatory responses.

CONCLUSION

The role and relevance of IRF5 in immune cell dysfunction in the context of autoimmune disease and cancer progression has become a hot topic for research in recent years. However, despite our growing knowledge of functions for IRF5 in APCs, our knowledge on the role of T cell-intrinsic IRF5 function is still lacking. Most of the literature published on potential roles for IRF5 in T cells is confounded by the dysregulation of other upstream immune cell signaling pathways in the *in vivo* setting of an *Irf5*$^{-/-}$ mouse. The CD4+ T cell-specific *Irf5* KO model attempted to address this and, in the context of CD3/CD28 TCR stimulation with IL-12, showed no defects in IFN-γ production (54). However, preliminary work from our lab utilizing *RAG2*$^{-/-}$ mice as recipients of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ T cells reveals a stimulus-dependent T cell-intrinsic defect that drives aberrant immune cell responses which, in the context of the hypothesized TLR driven IRF5 pathways in T cells, rather than rejecting previous work, compliments their findings (data not shown). The generation and characterization of new T cell-specific conditional *Irf5* KO mice, combined with pathway-specific immune challenges, will help to delineate Irf5 intrinsic function in T cells. For example, to study an intrinsic role for Irf5 in Th17 cells, *Irf5*-floxed mice would be crossed to IL17(A/F)-cre mice to generate Th17-specific *Irf5* conditional KO mice. A number of T cell-specific cre-reporter strains are currently available that would help prove or disprove the presented hypotheses.

In the clinical realm, SLE is characterized by a heterogeneous patient population. In each patient, the disease shares several common characteristics, but ultimately has a unique landscape and response to treatment. This is likely driven by a "multi-hit" scenario where dysfunction or dysregulation of a single (or multiple) master regulatory factor, like IRF5, will predispose individuals to developing a specific brand of immune dysregulation with many shared pathological characteristics (112). However, ultimately the path of development and resulting severity of the disease is determined by the addition of other risk allelic variations, thereby leading to the unique signature characterizing individual autoimmune conditions. This also explains the as-of-yet undefined and heterogeneous pathway-specific triggers that lead to disease development in a perfect storm of self-perpetuating dysregulated pathway activation, characterized by aberrant cytokine production. Ultimately, the goal in effective therapeutic development is to find the most specific target that ameliorates the greatest number of disease phenotypes with the fewest off-target effects. In order to accomplish this, we need a detailed understanding of the pathways that govern each immune cell implicated in disease pathogenesis. Targeting the inflammatory cytokines themselves is a difficult and non-specific therapeutic option, although early clinical trials of low dose IL-2 administration have shown some promise in patients with treatment-resistant SLE (113). However, the list of "T cell" therapeutics for autoimmune disease is brief, and many of them [i.e., secukinumab, ixekizumab, broalumab, ustekinumab, iberdomide, AMG 570 targeting ICOS-L (NCT04058028)] have either yet to be proven efficacious in the treatment of SLE, are still in early clinical trials, or broadly target the functions of other immune cells (114–116). As a result, targeted delivery of therapeutic molecules to specific immune cell subsets that drive the dysregulated release of either pro-or anti-inflammatory cytokines is the future of effective personalized treatments for autoimmune disease.

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Defining the Threshold IL-2 Signal Required for Induction of Selective Treg Cell Responses Using Engineered IL-2 Muteins

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Among all T and NK cell subsets, regulatory T (Treg) cells typically respond to the lowest concentrations of IL-2 due to elevated surface expression of the IL-2R alpha chain (IL2RA; CD25) and the high affinity IL-2 receptor (IL-2R) complex. This enhanced sensitivity forms the basis for low-dose (LD) IL-2 therapy for the treatment of inflammatory diseases, where efficacy correlates with increased Treg cell number and expression of functional markers. Despite strong preclinical support for this approach, moderate and variable clinical efficacy has raised concerns that adequate Treg selectivity still cannot be achieved with LD IL-2, and/or that doses are too low to stimulate effective Treg-mediated suppression within tissues. This has prompted development of IL-2 variants with greater Treg selectivity, achieved through attenuated affinity for the signaling chains of the IL-2R complex (IL2RB or CD122 and IL2RG or CD132) and, consequently, greater reliance on high CD25 levels for full receptor binding and signaling. While certain IL-2 variants have advanced to the clinic, it remains unknown if the full range of IL-2R signaling potency and Treg-selectivity observed with low concentrations of wildtype IL-2 can be sufficiently recapitulated with attenuated IL-2 muteins at high concentrations. Using a panel of engineered IL-2 muteins, we investigated how a range of IL-2R signaling intensity, benchmarked by the degree of STAT5 phosphorylation, relates to biologically relevant Treg cell responses such as proliferation, lineage and phenotypic marker expression, and suppressor function. Our results demonstrate that a surprisingly wide dynamic range of IL-2R signaling intensity leads to productive biological responses in Treg cells, with negligible STAT5 phosphorylation associating with nearly complete downstream effects such as Treg proliferation and suppressor activity. Furthermore, we show with both *in vitro* and humanized mouse *in vivo* systems that different biological responses in Treg cells require different minimal IL-2R signaling thresholds. Our findings suggest that more than minimal IL-2R signaling, beyond that capable of driving Treg cell proliferation, may be required to fully enhance Treg cell stability and suppressor function *in vivo*.

Keywords: IL-2, mutein, regulatory T, Treg, tolerance, inflammation, autoimmunity

INTRODUCTION

Produced primarily by activated T cells, IL-2 influences critical aspects of the immune response and homeostasis. IL-2 serves dual opposing functions; it potently amplifies proliferative responses of effector T (Teff) and natural killer (NK) cells, while regulating immune homeostasis by driving regulatory T (Treg) cell proliferation, differentiation, and function [review by Abbas et al. (1)]; and both axes have been leveraged to treat human diseases. In cancer patients, high-dose IL-2 therapy enhances Teff and NK cell mediated tumor cell killing (2). More recently, low-dose IL-2 therapy has been tested in inflammatory and autoimmune diseases where Treg expansion and increased expression of functional markers have been correlated with disease improvement (3–7). The efficacy of the low-dose IL-2 therapy has been attributed to the fact that Treg cells exhibit exquisite sensitivity to IL-2 (8) compared to other cell types (9–11), and thus, Treg cells preferentially respond when IL-2 availability is limited. However, the therapeutic dose range is narrow, as doses that induce more robust Treg cell responses simultaneously drive Teff and NK cells activity, which can reduce efficacy or lead to disease exacerbation and/or toxicity (3, 4, 7, 12).

Efforts to widen the therapeutic window of low-dose IL-2 therapy in recent years have focused around molecular engineering to increase the selectivity of IL-2 on Treg vs. Teff and NK cells. Predominantly, the engineered versions of IL-2 with increased selectivity for Treg cells possess attenuated binding and/or activity toward the IL2RB chain or IL2RG chain (13–16) which increases dependence on CD25 for generating stable interactions with the IL2RBG signaling chains, thereby enhancing selectivity for cells that express higher levels of CD25, such as Treg cells. Weaker activity also restrains the undesirable effects on Teff and NK cells over a wider concentration range (17). Similarly, IL-2:IL-2 Ab complexes that generate an attenuated IL-2 signal have been reported to demonstrate increased Treg cell selectivity (18).

The critical role of IL-2 in regulating Treg cell number and function is supported by human genetic studies and mouse models that lack various components of the IL-2 and IL-2R pathway. For example, the phenotype of mice lacking the expression of IL-2, or IL2RA or IL2RB chain (19, 20), or the downstream transcription factor STAT5 (21), recapitulates a wide range of the defects observed in the Foxp3 loss-of-function *scurfy* strain and Foxp3-deficient mice that lack functional Treg cells (22–25). In the absence of the IL-2 signal, Treg cell numbers are reduced (but not completely absent), they express reduced levels of Foxp3 and other phenotypic and activation markers, and they lose their suppressor function, which result in a fatal lymphoproliferative and autoimmune disease. In people, IL2RA deficiency (26–28) or STAT5B gene mutations (29) has been correlated with diseases that manifest aspects of autoimmunity, and additionally, allelic variants of the IL-2 or IL-2R or downstream genes have been identified in association with increased risks for autoimmune inflammatory diseases [review in Abbas et al. and Humrich et al. (1, 30)]. In further support, reduced IL-2 production or IL-2R signaling has been observed in human patients with autoimmune diseases such as type 1 diabetes (T1D) [review by Long et al. and Hull et al. (31, 32)] and systemic lupus erythematosus (SLE) (30). Low-dose IL-2 treatment is aimed to remedy such a proximal deficit and to further boost the IL-2-dependent effects on Treg cells, the primary outcome being the expansion in number and possibly an enhancement of their suppressive function.

As mice that completely lack the expression of IL-2 or its receptor still develop Treg cells (20, 33, 34), it is thought that cytokines other than IL-2 (e.g., IL-15) that can activate STAT5 can compensate and promote survival and expansion during early Treg differentiation (21), or that certain aspects of early Treg cell differentiation do not require IL-2. The fact that fatal disease develops in these mice suggests that, even though present, these Treg cells do not behave as effective tolerance mediators. Furthermore, ablation of IL-2R selectively in mature Treg cells results in a similar fatal lymphoproliferative inflammatory disease observed in mice that completely lack Treg cells (33, 34), indicating that continuous IL-2 signal is required to maintain mature Treg function *in vivo*. Similar disparity has been observed in human patients, where Foxp3+ Treg cells exist but are insufficient at controlling pathogenic inflammation (35). In some cases, Treg cells from these patients display reduced sensitivity to IL-2 stimulation (36–38), suggesting that Treg cells lose their functional capacity in the absence of a certain threshold level of IL-2 signaling. These data suggest that biological responses of Treg cells are sensitive to different levels of IL-2 signal. For example, one can hypothesize that only minimal IL-2R signals are required to maintain a normal Treg population through modest proliferative and survival signals, while more robust IL-2R signals are required to support maximal suppressor function(s) and maintain Treg stability in inflammatory settings.

With an interest in engineering an attenuated IL-2 that would preferentially bolster Treg cell number as well as function in clinical settings, we hypothesized that variable IL-2 signaling potency would contribute differentially to a number of key biological responses in human Treg cells. We generated a panel of engineered IL-2 molecules with mutations (referred to as IL-2 muteins) that impact binding to IL2RB and/or CD25 and evaluated how Treg and non-Treg cells responded to attenuated IL-2 signal. Using STAT5 activation as a quantifier of the proximal IL-2R signal, we compared Treg vs. non-Treg cell responses to assess relative selectivity of these molecules and further attempted to define the threshold IL-2 signal required to trigger meaningful biological responses in Treg and non-Treg cells. Our data show that human Treg cells can tolerate a significant degree of attenuation in IL-2 signal for certain biological responses. Nonetheless, the key Treg cell responses that collectively contribute toward their effectiveness as an immune suppressor were quantitatively dependent on IL-2 signal, indicating that IL-2 is a requisite driver of mature human Treg cell function. In contrast, non-Treg T cells do not tolerate attenuated IL-2 signals, providing an explanation for how attenuated IL-2 muteins increase Treg-to-non-Treg selectivity. In addition, our results suggest that Treg cell proliferation,

activation marker expression, and suppressor function are sensitive to different threshold levels of IL-2R signaling, indicating that the requirements for IL-2 signal in Treg cells are heterogeneous.

RESULTS

Structure-Based Design of IL-2 Muteins With Attenuated Interactions to CD25 and/or IL2RB

To explore the impact of weakened interaction of IL-2 with its receptor, mutations were designed at three interfaces. The first approach was to attenuate interactions with IL2RB directly by altering IL-2 at the core interface in the IL-2 A and C helices (**Figure 1**). The second was to slightly attenuate binding to CD25 at the interface edge. The third was focused on removal of a highly surface exposed methionine that shows no contact to any of the three receptors to improve manufacturability. Combinations of mutations were made between different IL2RB attenuation mutations to further decrease potency, between IL2RB and CD25 contact residues to mix receptor attenuation, and with IL2RB and methionine mutations for attenuation and manufacturability. Structural analysis of the IL-2 cytokine-receptor quaternary complex crystal structure (PDB 2B5I) was performed using Pymol. The structural analysis along with data from single mutation analysis from previous studies (39) were the basis for the following designs. The helices A and C, which form the IL-2 interface with IL2RB, were mutated at 4 positions as single or double combination substitutions. The histidine at position 16 (H16) was mutated to longer charged residues E or R to introduce steric repulsion due to a lack of space available in the histidine pocket. The aspartate at position 20 (D20) was mutated to disrupt electrostatic and Van der Waals interactions by substitution with alanine, or increase steric repulsion by mutation to tryptophan, respectively. The loss of binding was expected to be stronger with the D20W mutation than the D20A, therefore double attenuation mutations were not made with D20W. Weakening of the IL-2:IL2RB interface at position N88 to either D (13) or K substitutions disrupts a bidentate hydrogen bonding pair. Mutation of the valine at position 91 (V91) to small polar serine, positively charge lysine, or negatively charged aspartate was designed to create a weak or moderate attenuation as it is adjacent to the core interface. Mutations predicted or previously observed to impart minor impact were combined to produce stronger attenuation in variants such as D20A/H16E, D20A/H16R, or V91K/D20A. A single mutation of E61Q was tested to partially disrupt the CD25 interface by reduction of an electrostatic interaction with lysine from CD25. The mutation of M104 to T, L, or V was designed to improve manufacturability by removing an oxidation site. The mutated IL-2 variants were fused to the Fc portion of human IgG1 containing an N297G mutation, which abrogates the Fc effector functions. The resulting molecule was bivalent for IL-2 which could result in avidity interactions with IL-2R.

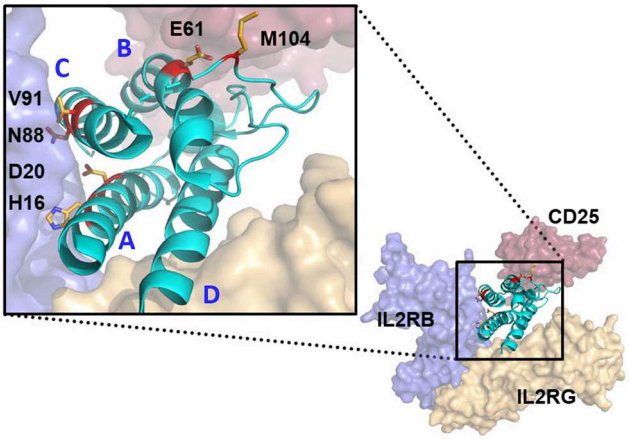

FIGURE 1 | Structure modeling of hulL-2:hulL-2 trimeric receptor complex and the amino acid residues mutated to attenuate the interaction with the various receptor chains. Model was generated from Pymol based on 2B5I pdb of the IL-2 co-crystal structure. CD25, IL2RB, and IL2RG are displayed as red, blue, and gold, respectively. IL-2 ligand is represented in Cyan with residues mutated in the study with side chains displayed and colored in red. Helices (A–D) are indicated in blue. IL-2 mutations that attenuate IL2RB are surface exposed residues which directly contact IL2RB.

The Mutein Panel Induces a Broad Spectrum of IL-2 Activity Measured by STAT5 Activation

IL-2 induces an intracellular signal via the heterodimeric receptor complex composed of IL2RB and IL2RG [review by Taniguchi and Minami (40, 41)]. CD25 can bind to IL-2 at low affinity by itself but does not induce a signal. However, inclusion of CD25 converts the intermediate affinity IL2RB:IL2RG heterodimeric receptor complex to a high affinity heterotrimeric receptor complex. Binding of IL-2 to the receptor activates JAK1 and JAK3 tyrosine kinases that are associated with the cytoplasmic tails of IL2RB and IL2RG chains, respectively. Activated JAK kinases phosphorylate tyrosine residues within the cytoplasmic tails of IL2RB and IL2RG, which serve as docking sites for downstream signaling molecules. Recruitment and activation of STAT5 is considered a critical event in IL-2 signaling (42, 43). Therefore, we evaluated relative potencies of our IL-2 muteins using STAT5 activation as the receptor proximal readout, measured by phospho STAT5 (pSTAT5) in a flow cytometry-based assay.

Since Treg cells constitutively express high levels of the high affinity IL-2R (8, 9) and are considered the most sensitive responders to IL-2 (9, 44, 45), we rank-ordered the muteins based on their activity on Treg cells (gating shown in **Supplementary Figure 1A**) measured by pSTAT5. As shown in **Figure 2A**, the dose response curves of the muteins in our panel show a wide range of activities, with varying degrees of attenuation compared to wild type IL-2. We compared the calculated EC50 values based on both the percent pSTAT5-positive cells and the mean pSTAT5 levels (**Supplementary Figure 1B**) indicated by the median fluorescence intensity (MFI) and determined that the rank orders

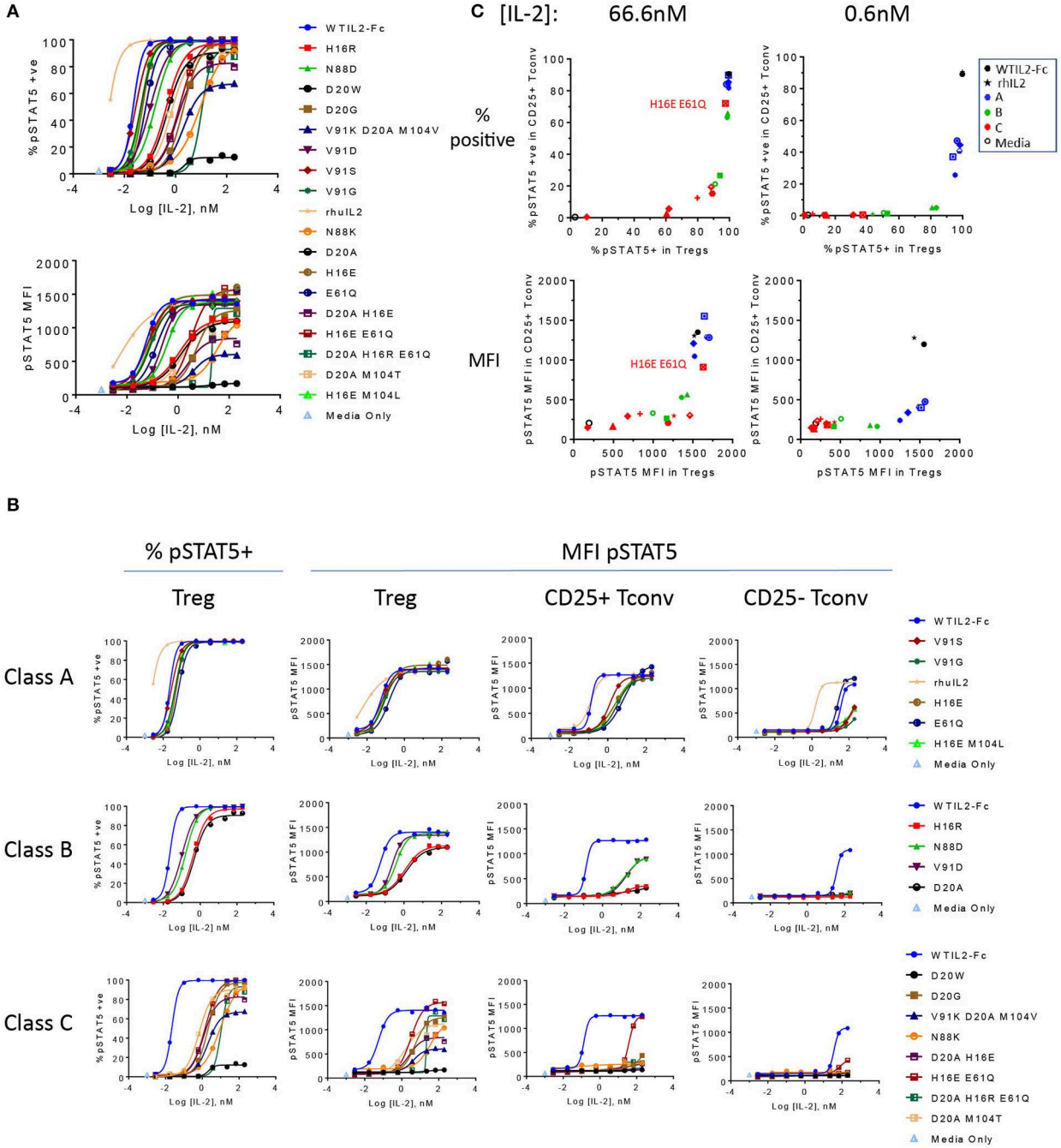

FIGURE 2 | The engineered IL-2 mutein panel induces a broad spectrum of pSTAT5 response. **(A)** Activity of the individual IL-2 muteins is represented by phospho STAT5 (pSTAT5) signal at increasing concentrations of IL-2 muteins in human whole blood cell assay. pSTAT5 responses are shown as both the percent (%) pSTAT5-positive cells in (top panel) and pSTAT5 MFI of (bottom panel) CD25hi Foxp3+ gated Treg cells (gating of an example plot shown in **Supplementary Figure 1**). Shown are representative plots of data from three donors. **(B)** The same pSTAT5 data shown in **(A)** are represented according to the potency-based classification to illustrate the impact of attenuation on various cell populations. Both the percent positive and pSTAT5 MFI for Treg gated populations are shown. In addition, pSTAT5 MFI data are shown for CD25+ Tconv, and CD25– Tconv cells. **(C)** The activity of the muteins on CD25+ Tconv cells vs. Treg cells are shown in 2D plots. Percent pSTAT5-positive and pSTAT5 MFI readouts at mutein concentrations 66.6 and 0.6 nM are shown, color coded to represent wildtype IL-2 as the Fc fusion (WTIL2-Fc, black circle), free recombinant cytokine (rhIL2, black star), class A (blue), B (green), C (red) muteins, and media only control (black open circle).

of the mutein potencies were similar. To better understand how the attenuation impacted downstream biological responses, and to simplify the comparison amongst different muteins, we grouped them into three classes using arbitrary cutoff points established based on their activity relative to wildtype IL-2 (**Table 1**).

TABLE 1 | Mutein classification based on the pSTAT5 MFI EC50 values.

Class	Muteins	AVG EC50 (nM), $n = 3$	STDEV EC50	AVG dMFI at 66.7 nM, $n = 3$
A	rhIL2	0.008	0.0097	1455.3
	WT	0.057	0.0054	1501.3
	H16E M104L	0.062	0.0257	1225.3
	H16E	0.067	0.0244	1277.7
	V91S	0.089	0.0393	1154.7
	E61Q	0.117	0.0300	1680.3
	V91G	0.153	0.0768	1055.0
B	V91D (A/B)	0.367	0.1415	1000.0
	N88D (A/B)	0.418	0.0564	1032.7
	H16R	1.168	0.1601	860.3
	D20A	1.205	0.8115	939.0
C	D20A H16E	1.596	0.8670	697.7
	D20A M104T	1.666	1.1530	846.7
	H16E E61Q	2.343	0.8809	1258.3
	V91K D20A M104V	4.763	0.6466	430.0
	D20G	6.244	1.9392	825.3
	D20A H16R E61Q	20.720	7.0428	994.3
	D20W	20.924	16.1745	17.5
	N88K	23.315	14.1916	688.7

The average EC50 values were calculated from non-linear regression analysis of IL-2 mutein dose response curves generated by pSTAT5 MFI readout. The average values with standard deviation (STDEV) were calculated from three donors. Calculated dMFI values at [IL-2] = 66.7 nM are also included.

We defined class A as the group of muteins whose potencies (determined by pSTAT5 MFI EC50 values) are within 5-fold that of wildtype IL-2 and thus these muteins are only slightly attenuated. Class B muteins represent moderately attenuated muteins, where the EC50 values are between 5- and 25-fold higher compared to wildtype. Finally, class C muteins are highly attenuated, as their EC50 values are 25-fold or higher than that of the wildtype IL-2. The dose response curves of the muteins classified in this manner are shown in **Figure 2B**. As defined, both class B and class C muteins showed significant shift in EC50 values compared to wildtype IL-2, but class C muteins additionally demonstrated varying degrees of attenuation in maximal response (Rmax), as indicated by the plateau in dose response curves at high IL-2 concentration range. Attenuation of activity by EC50 and Rmax was not always linked, since there are muteins with >25-fold shift in EC50 but are still able to induce close-to-wildtype Rmax at high concentrations. Muteins such as D20G, D20A M104T, and D20A H16R E61Q represent this category. The weakest mutein (D20W), showed very little activity, indicated by its ability to induce activity in no more than 10% of the cells and barely detectable increase in pSTAT5 MFI. Thus, our mutein panel captures a wide spectrum of IL-2R signal, from wildtype level to almost complete lack of activity at a concentration as high as 200 nM *in vitro*.

Attenuation of IL-2R Signaling Asymmetrically Impacts Treg and Non-Treg Cells

Interestingly, class A muteins, which showed very little attenuation in pSTAT5 response in Treg cells, demonstrated significant attenuation in CD25+ Foxp3– CD4 T cells (designated CD25+ Tconv in this paper for simplicity) as shown by the shift in MFI dose response curves (**Figure 2B**). Class B and C muteins showed even greater attenuation of activity in these cells. Although CD25 does not directly trigger intracellular signaling events, it can enhance the on-cell IL-2 activity by capturing IL-2 in solution and stabilizing the high affinity heterotrimeric receptor. Thus, we chose to compare the IL-2 activity on Foxp3+ (Treg) and Foxp3– CD4 T (Tconv) cells that are gated for positive CD25 expression. Nonetheless, CD25 levels on *ex vivo* Treg cells are significantly higher than those on unstimulated CD25+ Tconv cells (CD25 MFI, **Supplementary Figure 1A**) and as a result, a mutein's affinity to CD25 and/or its ability to aid in or hinder the assembly of the trimeric receptor may additionally impact its relative activity in Treg vs. non-Treg cells. To further narrow the differences in CD25 levels for this comparison, we also compared pSTAT5 response in CD25lo Treg cells gated to more similarly match the CD25 level on CD25+ Tconv cells (CD25+/lo Tconv in **Supplementary Figure 3**), but the differences in sensitivity of Treg and CD25+ Tconv cells persisted (**Supplementary Figure 3**). Additionally, to evaluate the signaling capacity of the muteins independently of their affinity for CD25, we evaluated the pSTAT5 response in cells that are negative for CD25. pSTAT5 responses in these cells, shown here by pSTAT5 data from CD25– Tconv cells (**Figure 2B**) and NK cells (**Supplementary Figure 2**), are significantly weaker compared to the CD25+ gated cells, and these represent IL-2 mutein activities generated solely through IL2RB and IL2RG We also note that the recombinant IL-2 showed stronger activity than the wildtype IL-2 in our molecular format, which may be due to aggregation (reported on the Proleukin label) and a resulting increase in avidity.

To further visualize how the attenuation impacted the muteins' activities in CD25+ Tconv cells compared to Treg cells, we plotted the percent pSTAT5+ and pSTAT5 MFI responses detected in CD25+ Tconv vs. Treg cells at two concentrations, at 66.6 nM and at 0.6 nM. The higher concentration corresponds to a concentration where most muteins reach their maximal response in Treg cells and therefore we consider it to represent a concentration of IL-2 that saturates the trimeric receptors on the cell surface. The lower concentration is 100-fold lower than this saturating concentration and for many muteins stands in the linear dose range. As indicated by the percent pSTAT5+ dose response data (**Figure 2B**), class A muteins are able to generate response in 100% of Tregs and close to 100% of CD25+ Tconv cells at saturation, while some of the class B and class C muteins are not able to induce response in 100% of cells even at the highest concentration tested, confirming that attenuation of STAT5 activation correlates with a significant loss in affinity to the receptor for these classes of muteins. **Figure 2C** demonstrates that the attenuation impacts the mutein activities in CD25+ Tconv vs. Treg cells non-linearly. For example, class A muteins induced maximal activity in both Treg and CD25+ Tconv cells at the high concentration, while the activity declined disproportionately at

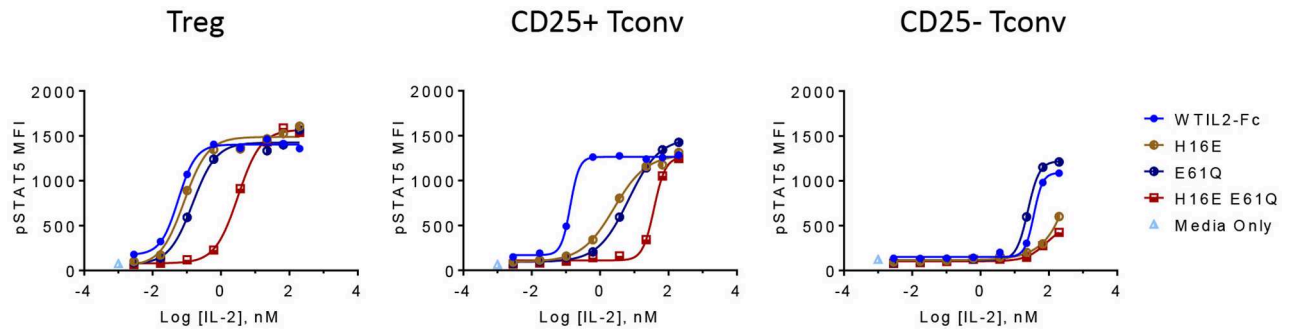

FIGURE 3 | CD25 and IL2RB attenuation synergistically impacts IL-2 mutein activity and selectivity in CD25+ cells. The activity of IL-2 muteins containing a single mutation that slightly reduces affinity to CD25 (E61Q) or to IL2RB (H16E), or both (H16E E61Q), is shown as pSTAT5 MFI at increasing IL-2 mutein concentrations in CD25hi Treg cells, CD25+ Tconv cells, and CD25– Tconv cells. Shown are representative of three donors.

the lower concentration. At this non-saturating concentration, the muteins activity dropped dramatically in CD25+ Tconv while maintaining near maximal activity in Treg cells. Class B muteins displayed different behaviors at the high and the low concentrations. At saturation, these muteins showed significant attenuation selectively in CD25+ Tconv cells while they were still able to generate near maximal signal in Treg cells. At the lower concentration, their activity on Treg cells showed a range of attenuation while the activity in CD25+ Tconv cells remained maximally attenuated. Most of the class C muteins showed significantly attenuated activity in Treg and CD25+ Tconv cells at both concentrations. An exception was the H16E E61Q mutein, which contained a CD25 association mutation and showed a near-wildtype level of pSTAT5 response at the high concentration. These results show that the attenuation differentially impacts Treg vs. non-Treg cell subsets. IL-2-induced pSTAT5 response declined disproportionately faster in CD25+ Tconv cells with the attenuation, indicating that these cells exhibit heightened sensitivity to loss of IL-2 signal compared to Treg cells, a difference that was also observed for Treg and CD25+ Tconv cells gated for similar CD25 levels.

TABLE 2 | Relative binding affinity of IL-2 muteins to CD25 and IL2RB.

	CD25	IL2RB	
Mutein	**EC50, nM**	**% of WT binding**	**Class**
V91K D20A M104V	6.0	1.1	C
H16R	7.2	2.9	B
V91D	11.2	1.2	B
V91G	13.8	8.5	A
WTIL2-Fc	**19.7**	**101**	A
D20W	43.1	0.9	C
D20A H16R E61Q	156.9	–3.7	C
D20G	175.0	4	C
H16E E61Q	310.8	1	C
E61Q	651.9	98	A

A subset of the mutein panel was evaluated for both CD25 and IL2RB relative binding activity using alternate assays. For CD25, EC50 values were determined from titrations of individual muteins against immobilized receptor by SPR binding assay as outlined in the methods. For IL2RB, relative binding affinity was approximated via the steady state binding RU level observed at a nominal mutein concentration of 1,000 nM where the values are reported as a percentage of the response observed with wildtype IL-2 (WTIL2-Fc) binding to immobilized IL2RB. WTIL2-Fc values are highlighted in bold as reference.

Attenuation of Affinity to CD25 and IL2RB Synergistically Impacts Activity in CD25+ Cells

We measured the affinity of a subset of the muteins to CD25 and IL2RB to confirm our design rationale and to gain an insight into their activity and selectivity. As mentioned previously, most of the muteins contain a single or combination of mutations that are designed to disrupt the interaction with IL2RB. The muteins containing E61Q were designed to disrupt the interaction with CD25 to evaluate the additive impact on the IL-2 signal. Consistent with this design goal, the muteins that contain E61Q exhibit significant increase in their EC50 values (156.9–651.9 nM), suggesting weaker affinity to CD25. In contrast, muteins that exclude E61Q demonstrate similar affinity to CD25 as indicated by their EC50 values (6.0–43.1 nM, **Table 2**) obtained by SPR that was within the range observed for wildtype IL-2 (19.7 nM). Wildtype IL-2 binds to IL2RB only very weakly and in cells is far more likely to bind the IL2RB:IL2RG heterodimer rather than IL2RB alone. Nonetheless we evaluated binding activity of the muteins to IL2RB to confirm the attenuation. Due to the very weak signal measurable for IL-2:IL2RB interaction in this assay, we assessed binding of the muteins to IL-2RB relative to that of wildtype IL-2 (WTIL2-Fc) at a high fixed concentration (1,000 nM) (**Table 2**). Consistent with our design goal, E61Q by itself had very little impact on affinity to IL2RB, while all muteins containing IL2RB-attenuating mutations showed significant loss of binding. Among them, the class A muteins retained higher binding activities (V91G), while class B and some of the class C muteins retained trace amount of binding. The weaker class C muteins showed complete or almost complete loss of activity. These data confirm that, consistent with our design goal, IL-2 mutein activity correlated with its affinity to IL2RB. More importantly, these data show that Treg cells can induce

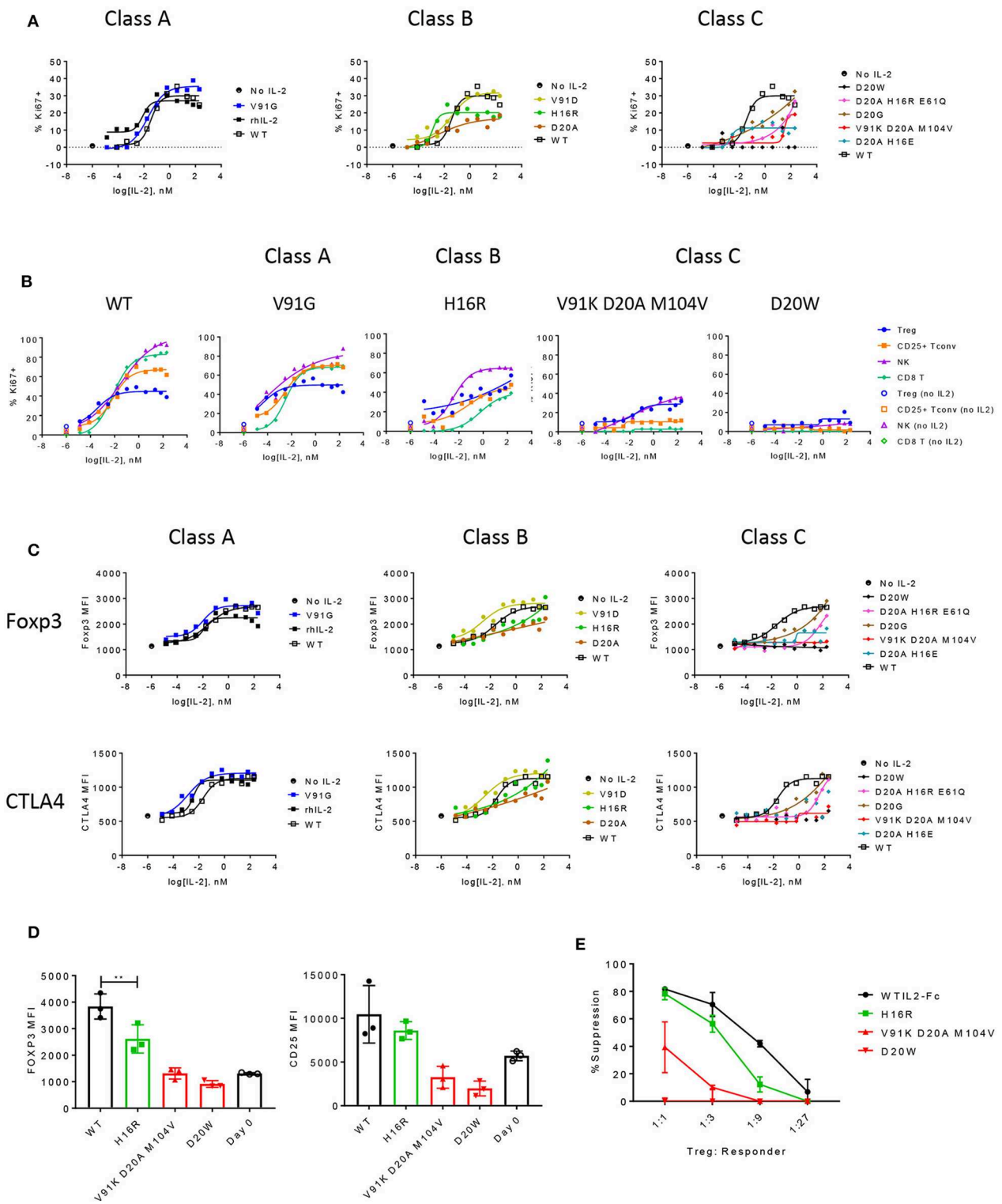

FIGURE 4 | The functional responses of Treg cells are quantitatively sensitive to attenuated IL-2 signal. **(A)** Proliferation of Treg cells in response to select muteins from each class was measured in human PBMC assay and shown as percent Ki67-positive cells in CD25+ Foxp3+ CD4-gated Treg cells. Shown are data representative of four donors. **(B)** Proliferative responses measured as percent Ki67 positives of Treg, CD25+ Tconv, NK (CD56+ CD3–), and CD8 T cell gated subpopulations to increasing concentrations of wildtype or IL-2 muteins are compared in a total PBMC assay, for select muteins from each class. Shown are representative of data from four donors. **(C)** IL-2 mutein activity on induction of Foxp3 and CTLA4 in Treg cells are shown, represented as MFI of Foxp3 or CTLA4 in Foxp3-positive and

(Continued)

FIGURE 4 | CTLA4-positive gated Treg cell populations, respectively. **(D)** Treg phenotype before and after stimulation. Purified human Treg cells were stimulated with anti-CD3 and wildtype or IL-2 mutein at 66.7 nM for 3 days and analyzed for Foxp3 and CD25 expression. Day 0 unstimulated Treg cells analyzed at the same time to show baseline expression of these markers. Each color represents the mutein class or day 0: wildtype (black closed circles), class A (blue), class B (green), class C (red), and day 0 (black open circles). Shown are combined data from three different donors. ** represents *p*-value of 0.005 from a one-way ANOVA analysis. **(E)** Treg suppression assay. Purified human Treg cells that were pre-stimulated with anti-CD3 and IL-2 mutein were co-cultured with purified CD8 T cells from an unmatched donor at various ratios. CD8 T cells were stimulated with CD3+CD28 activation beads and Treg-mediated suppression was measured by activation marker induction on CD8 T cells on day 1. These values were converted to percent suppression, each point representing the average of values from two donors. Error bars indicate standard deviation.

relatively normal pSTAT5 response despite significant loss in binding to IL2RB, presumably due to a significant contribution of CD25 in the formation of the receptor complex that facilitates downstream signaling events and STAT5 activation.

The CD25-attenuating E61Q mutation by itself had only a minor impact on overall activity as shown by the pSTAT5 EC50 and Rmax values in Treg cells (**Figure 3**), indicating that the levels of CD25 on Treg cells are high enough to tolerate partially reduced CD25 affinity in IL2-Fc homodimers. In CD25+ Tconv cells, with reduced CD25 levels, E61Q induced an attenuated pSTAT5 signal compared to wildtype IL-2, while in Tconv cells that were gated to exclude CD25-positive cells, E61Q retained activity similar to wildtype. This indicates that the attenuating effect of E61Q is dependent on and is sensitive to CD25 levels. In cells that express CD25 (Treg and CD25+ Tconv), E61Q exerted a significantly additive effect on attenuation when combined with an additional mutation that disrupts the interaction with IL2RB, since the potency of the mutein containing double mutations, H16E E61Q, is more than 30-fold decreased compared to that of the H16E single mutein. In contrast, in cells that lack CD25, H16E E61Q showed the same activity profile as did H16E single mutein. These results suggest that CD25– and IL2RB-directed mutations can additively attenuate IL-2 signal in CD25+ cells. D20W, which induced barely any detectable pSTAT5 signal, still exhibited normal affinity to CD25 suggesting that this mutein retained protein stability and integrity.

Treg Cells Tolerate Significant Loss of IL-2 Activity in Generating Biological Responses

Using *in vitro* assays, we evaluated how effectively these IL-2 muteins induced distal biological responses and attempted to define a threshold IL-2 signal that would selectively induce cell proliferation, upregulate activation markers, and enhance suppressive activity in Treg cells. We performed proliferation assay with human PBMC in the presence of titrating doses of IL-2 muteins and measured the expression of the proliferation marker Ki67. As shown in **Figure 4A**, class A mutein V91G induced similar Ki67 response as did wildtype IL-2 in Treg cells. Among the class B muteins, V91D, the most potent mutein in this class, behaved more like class A muteins and showed very similar activity as the wildtype control in all measurements from this assay (**Figure 4C**). For this reason, we re-classify V91D and N88D (**Supplementary Figure 4**) as class A muteins (shown as A/B in **Table 1**) for subsequent analyses. H16R, with a 20-fold reduction in potency, did show a significant loss in activity in inducing Treg cell proliferation. These results suggest that Treg cells retain full response to IL-2 with up to 6–20-fold loss in potency. The rest of class B and class C muteins showed varying degrees of reduced response across a wide concentration range compared to wildtype IL-2. The weakest mutein, D20W, did not induce any detectable Ki67 expression in Treg cells.

In this same assay, we also evaluated the IL-2-induced proliferative responses of additional cell subsets to determine whether CD25+ Tconv, CD8 T, and NK cells showed similar sensitivity to IL-2 attenuation as did Treg cells. Wildtype IL-2 and all class A muteins induced higher proliferative responses in CD25+ Tconv, CD8, and NK cells compared to Treg cells at IL-2 concentrations higher than $\sim 10^{-2}$ nM (**Figure 4B** and data not shown), while inducing comparable or weaker response at the lower concentration range ($<10^{-2}$ nM). Consistent with the observation that the class A muteins generated weaker pSTAT5 response in non-Treg cells compared to wildtype IL-2, the muteins induced diminished Ki67 response in CD8 and NK cells (**Figure 4B**). Interestingly, class A mutein effect on CD25+ Tconv proliferation was not diminished compared to wildtype IL-2. We think that this may be due to an autocrine effect of endogenously produced wildtype IL-2 on pre-stimulated CD4 T cells, which may mask signaling deficit of a mildly-attenuated class A mutein. Class B muteins showed significantly reduced response in CD25+ Tconv and CD8 T cells to the extent that Treg cell response was similar to or better than non-Treg T cell responses. Interestingly, NK cells still proliferated better than Treg cells, even though the IL-2 mediated pSTAT5 response in NK cells is significantly weaker compared to Treg and other cell types (**Supplementary Figure 2**). One possible explanation for such a disproportionately sensitive response of NK cells is that other cell types present in this assay indirectly aid in NK cell proliferation, by producing additional cytokine(s) or by capture and transpresentation of IL-2 via CD25. Class C muteins showed further attenuation across all cell types, but most significantly in CD25+ Tconv and CD8 T cells, thus these muteins preferentially induced Treg cell proliferation even at high concentrations. D20W did not induce significant response in any of the cell subsets, consistent with the minimal pSTAT5 signal it induces. These data together suggest that CD25+ Tconv and CD8 T cells require higher IL-2 activity than do Treg cells, since even a modest degree of attenuation significantly reduced IL-2 mediated proliferation in these cells while exerting little impact on Treg cells.

Previously published studies have shown that Treg cell lineage and phenotypic markers such as Foxp3 and Helios and T

cell activation markers such as CTLA4, ICOS, and GITR, are expressed at high levels in Treg cells and correlate with stability of Treg cell phenotype and/or suppressive function [reviews by Rudensky (46) and Elkord (47)]. Importantly, Foxp3 expression is thought to be regulated directly by IL-2 (21, 48), thus this represents one mechanism by which IL-2 enhances Treg cell phenotype and function. Therefore, we evaluated the muteins' ability to increase the expression of Foxp3 and other markers associated with Treg cell phenotype and function. As shown in **Figure 4C**, wildtype IL-2 and class A muteins induced robust expression of Foxp3 and CTLA4 in an IL-2 dose-dependent manner, while class B and C muteins demonstrated varying degrees of attenuation. Some muteins in these classes were able to induce a nearly wildtype level of expression when pushed to high concentration range despite dramatic attenuation in potency indicated by EC50 values; others induced no significant response throughout the entire concentration range. Overall the muteins exhibited parallel activities toward Foxp3 and CTLA4 (and others, data not shown) induction. In this assay, where we could directly compare proliferation and phenotypic marker expression simultaneously, we observed that our weaker class C mutein (V91K D20A M104V) was unable to increase Foxp3 or CTLA4 expression at all, even though it was able to induce Treg cell proliferative response at the highest concentration. This suggests that the threshold IL-2 activity required to induce Treg cell proliferation may be lower compared to that for the Foxp3 or CTLA4 expression.

The ability of muteins to enhance Treg cell fitness to suppress immune response was assessed in an *in vitro* Treg suppression assay. In order to minimize the overpowering effects of wildtype IL-2 produced by activated T cells, we assessed early readout of T cell activation in CD8 T cells co-cultured with purified Treg cells that were first stimulated with IL-2 muteins. Due to the limited number of Treg cells that could be purified from each donor, we evaluated only a representative mutein from classes B and C and compared their effects against those induced by wildtype IL-2 or D20W mutein, which we used as a negative control. As shown in **Figure 4D**, Foxp3 and CD25 induction by IL-2 muteins in purified Treg cells correlated with the mutein activity as previously shown in the proliferation and pSTAT5 assays. Specifically, H16R, a class B mutein, induced attenuated expression of Foxp3 and CD25 compared to wildtype IL-2, while both class C muteins, V91K D20A M104V and D20W, resulted in no enhancement of expression of these key markers compared to day 0 when the Treg cells were isolated. Similar trends were observed for CTLA4 (**Supplementary Figure 5A**). We evaluated early activation response of allogeneic CD8 T cells in the presence of these pre-stimulated Treg cells and report that the muteins demonstrated variable degrees of attenuation compared to wildtype IL-2 in supporting Treg-dependent suppression (**Figure 4E**; **Supplementary Figure 5B**). Interestingly, H16R was nearly as effective as wildtype IL-2 in inducing full suppressive function despite the modest but significant attenuation it had shown in inducing Foxp3 and CTLA4 expression (**Figure 4D**; **Supplementary Figure 5**). Nonetheless, Treg cells stimulated in the presence of H16R showed weaker activity at lower Treg:CD8 T ratios indicating that the functional enhancement is sensitive to relative IL-2 activity. Showing a similar trend, V91K D20A M104V induced attenuated but significant level of suppression despite its inability to induce Foxp3, CD25, and CTLA4 expression in Treg cells.

To further assess the impact of attenuated IL-2 activity on maintaining gene expression associated with Treg cell stability and function, we performed Taqman assays for a panel of genes that have been shown to be induced downstream of activated STAT5 in Treg cells (33), including suppressor of cytokine signaling 2 (*socs2)*, cytokine inducible SH2 containing protein (*cish*), Ras homolog family member c (*rhoc*), vimentin (*vim*), *foxp3,* and CD25 (*IL2ra)*. As shown in **Figure 5A** (and extended panel in **Supplementary Figure 6**), the transcript levels of multiple genes increased in response to wildtype IL-2, with attenuated muteins showing reduced response commensurate with their pSTAT5 signal. Importantly, we observed that the two class C muteins failed to induce significant accumulation of *foxp3* transcripts compared to wildtype IL-2 or the class B mutein, consistent with their lack of activity in maintaining Foxp3 protein expression in activated Treg cells (**Figure 4D**). This result indicates that transcript levels of Treg-associated genes correlate quantitatively with IL-2 signal.

Stable expression of Foxp3 is associated with demethylation of Treg-specific CpG methylation sites in the first intron of the *foxp3* gene, referred to as Treg-specific demethylated region (TSDR) (49, 50). Since we observed that *foxp3* transcript levels diminished with decreased IL-2 activity, we evaluated whether IL-2 activity is required for continuous maintenance of demethylation in TSDR by performing bisulfite sequencing analysis of genomic DNA from purified Treg cells stimulated with wildtype IL-2 of IL-2 muteins. As indicated by the calculated C/T conversion rate, TSDR is highly demethylated in purified Treg cells compared to Tconv cells across multiple CpG sites on day 0 (**Figure 5B**, inset). After 36-h stimulation with or without IL-2 muteins, TSDR remained fully demethylated regardless of the IL-2 activity, since the two weak class C muteins, as well as untreated sample, showed comparable activity as wildtype IL-2 in this assay. These results indicate that the maintenance of demethylation state of TSDR in purified human Treg cells is relatively resistant to attenuation or even loss of IL-2 activity, at least in an *in vitro* setting in this time frame.

Induction of Treg Gene Signature Requires Stronger IL-2 Signal Than Do Other Treg Functional Responses

We generated correlation plots comparing the various measurements of biological responses against IL-2 mutein-induced pSTAT5 signal to interrogate whether a threshold IL-2 signal could be defined that leads to a detectable biological response. Since the biological assays were performed with pre-stimulated and rested PBMC, we evaluated pSTAT5 response of these cells with a subset of IL-2 muteins to establish that the muteins induced comparable pSTAT5 response on *ex vivo* cells (d0) as they did in stimulated and rested cells (d8) (**Supplementary Figure 7A**). The CD25 levels on Treg and CD25+ Tconv cells on day 8 were not vastly different from those

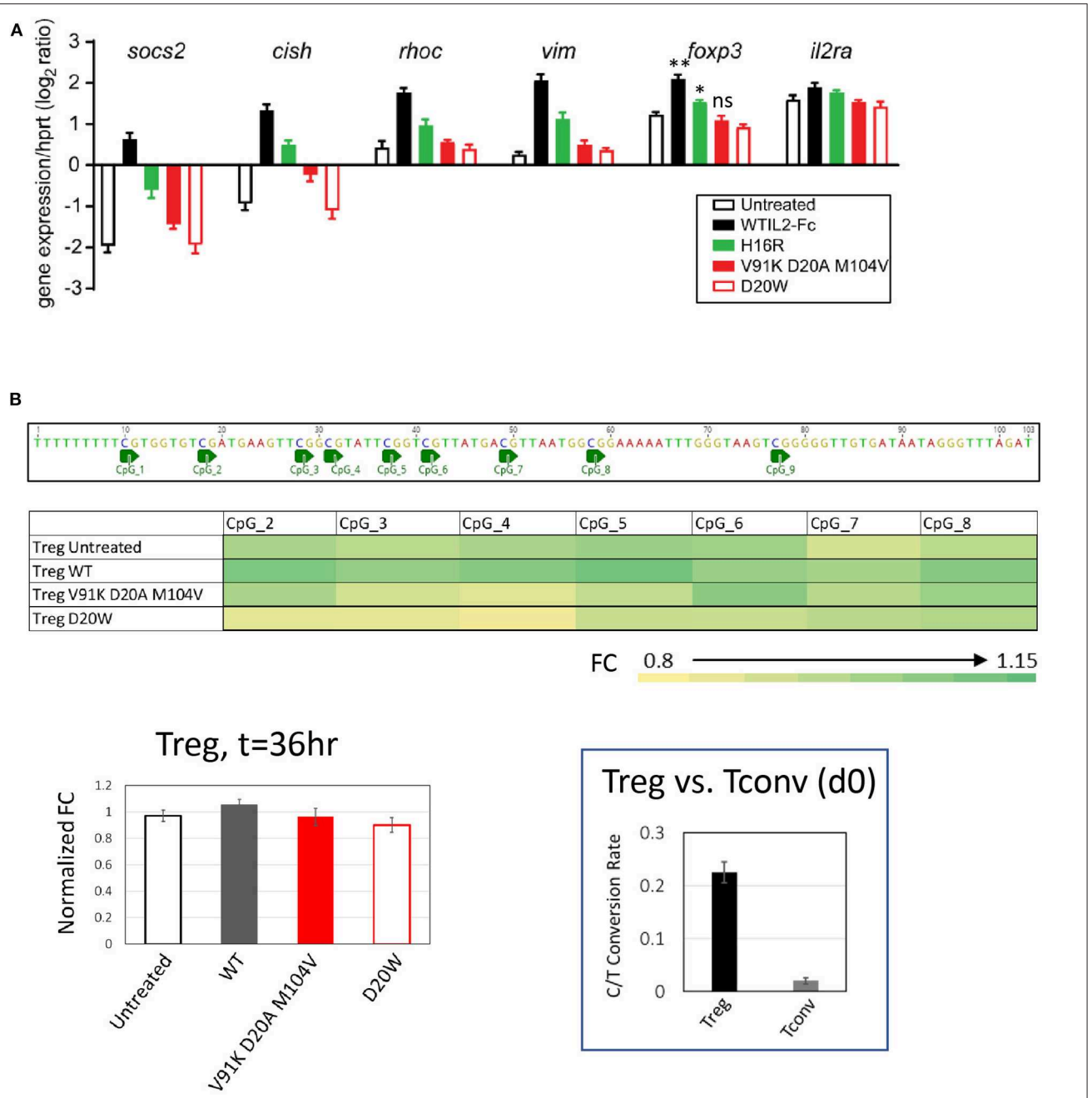

FIGURE 5 | Treg-associated gene response is quantitatively sensitive to attenuated IL-2 mutein activity. **(A)** Transcript levels of *socs2*, *cish*, *rhoc*, *vim*, *foxp3*, and *il2ra* were quantified by Taqman assay in purified human Treg cells stimulated with various IL-2 muteins for 36 h. The results represent the mean ± SD of relative expression values (log2 ratio) for the indicated genes normalized to hypoxanthine-guanine phosphoribosyl transferase (*hprt*) in two independent donors with three replicates each. For *foxp3*, paired *t*-test was performed for each treatment group against the untreated sample and two-tailed *p*-values are indicated. $^{**}p = 0.0076$; $^{*}p = 0.0165$. **(B)** Methylation status of *foxp3* CNS2 region in human Treg cells cultured in IL-2 muteins. Methylation status was quantified as C-to-T conversion rate after bisulfite treatment. The fold change (FC) values were calculated based on the C-to-T conversion rates at individual CpG sites and normalized to Day 0 sample. The average FC values from three donors are plotted into heatmap. The average FC values calculated from the combined CpG sites were plotted as bar graph to show IL-2 mutein dependent effects. The BS4 amplicon sequence and CpG positions were illustrated above the heatmap. Shown in the inset is the representation of the overall C-to-T conversion rates for purified Treg and Tcon cells on day 0.

on day 0 (**Supplementary Figure 7B**), thus we used the pSTAT5 readout on day 0 for the correlation analysis. As expected, we observed a positive correlation between the pSTAT5 signal and Treg cell proliferation, Foxp3 and CTLA4 expression throughout the range of different IL-2 mutein concentrations (**Figure 6A**). The IL-2 mutein activity also positively correlated with Treg cell

survival, as indicated by the strong correlation seen between the total Treg cell frequency (% Treg of CD4 T) vs. pSTAT5 MFI and between Treg cell frequency vs. % Ki67+ Treg cells (**Supplementary Figure 8**). This indicates that the enhanced Treg cell proliferation and survival in response to IL-2 muteins leads to increased Treg cell number. The correlation was stronger at higher concentrations where the attenuation of the pSTAT5 signal would be expected to primarily reflect attenuation of the mutein activity at maximal possible receptor occupancy for the individual mutein. At lower concentrations where the mutations' impact on receptor binding and total occupancy may additionally factor in, the correlation was not as strong. In this analysis, class A muteins showed dramatic losses in pSTAT5 signal across the concentration range (66.7–0.1 nM), but it did not result in a corresponding reduction in biological responses. In fact, V91D was able to induce near maximal Ki67, Foxp3, and CTLA4 expression, despite over 67% reduction (estimated by the difference in pSTAT5 MFI) of pSTAT5 signal compared to wildtype IL-2 at 0.1 nM. These analyses also confirmed that class B and C mutein activities were reduced compared to class A muteins, as indicated by both pSTAT5 and biological responses, but similarly to class A muteins, there was a significant dissociation between the attenuation of activity represented by pSTAT5 readout vs. the effects on biological readout. Thus, although pSTAT5 MFI signal decreased significantly from 66.6 nM, 0.62 nM, to 0.1 nM, similar levels of the Ki67, Foxp3, and CTLA4 expression were maintained across all concentrations for individual muteins. This suggests that Treg cells are able to respond productively downstream of STAT5, as defined by their ability to proliferate and upregulate activation markers, to a wide range of IL-2 activity and that they can tolerate a significant degree of attenuation in IL-2 activity.

We took a closer look at these correlation plots, focusing on the weaker muteins for their ability to induce biological responses at lower concentrations. At 0.62 and 0.1 nM, the weakest muteins were not able to enhance Foxp3 and CTLA4 expression, despite generating small but significant pSTAT5 response over the baseline, establishing a threshold pSTAT5 signal that is required for induction of these markers (**Figure 6A**). Interestingly, some of these same muteins still induced significant Treg cell proliferation in this concentration range, suggesting that the threshold IL-2 activity for Treg cell proliferation is lower.

Similarly, attenuated muteins were able to support the suppressor function of Treg cells in an *in vitro* assay, but these activities were attenuated compared to wildtype IL-2 and ranked consistently with their pSTAT5 response (**Figure 6B**). In this assay, V91K D20A M104V demonstrated significant activity toward sustaining Treg cell suppressor function without a concomitant upregulation of Foxp3 (**Figure 4D**) and other activation markers (**Supplementary Figure 5A**), suggesting that IL-2 may be able to enhance Treg cell function independently of its effects on at least a subset of the canonical Treg cell markers. Finally, we observed that all of the class B and C muteins induced notably skewed Treg:CD25+ Tconv cell responses with preferential Treg cell response across the IL-2 concentration range, which distinguished them from the class A muteins which, like wildtype IL-2, induced greater Teff responses (**Figure 6C**). Thus, the Treg:CD25+ Tconv selectivity appeared to correlate with the degree of attenuation in activity, rather than the specific residues that are mutated.

We further evaluated the ability of these muteins to induce Treg cell response *in vivo* using the humanized NSG (huNSG) mouse model, where human lymphocytes derived from the grafted human hematopoietic stem cells (huHSC) circulate in animals that lack their own lymphocytes. As shown in the study design schematic, we pre-treated huNSG mice with human gamma globulin to block non-specific binding of our molecules and dosed them twice with PBS, wildtype IL-2, or IL-2 mutein and analyzed the blood 4 days after the second dose (**Figure 7A**). We chose a low dose, at total of 1.5 μg, which we had determined with the wildtype molecule was a dose that induced a robust Treg cell expansion with minimal effect on Tconv and other non-Treg cells (data not shown), thus mimicking the effect of the low-dose IL-2 therapy in human patients. As shown in **Figure 7B**, wildtype IL-2 induced nearly a 5-fold increase in Treg cell number as indicated by the increased percentage of Foxp3+ CD4 T cells among total CD4 T cells, compared to the PBS-treated group. The two class A muteins showed a slightly reduced but still significant increase, while H16R, a class B mutein, showed a trend toward an increase but the effect was not statistically significant at this dose. D20G, a class C mutein, did not induce a response. These trends are conserved in the expression of Ki67 and Foxp3, as class A and B muteins showed a trend toward increased expression while the class C mutein showed responses that are comparable to the PBS-treated group. Importantly, wildtype IL-2, which robustly increased Treg cell number at this dose, showed a modest increase in Foxp3 expression. Thus, consistent with the *in vitro* data, Treg proliferative response appeared to be more sensitive to weak IL-2 signal than induction of Treg-associated gene expression *in vivo*. Furthermore, consistent with the general lack of effects of wildtype IL-2 at this low dose on Tconv cells, the attenuated muteins also did not show any effect on this population of cells (**Figure 7C**). Importantly, all muteins tested in this study also demonstrated reduced activity toward CD8 and CD16+ NK cells. These data further confirm our *in vitro* results that the Treg-to-non Treg selectivity is enhanced with attenuated IL-2.

DISCUSSION

Treg cells drive dominant tolerance by inhibiting inflammatory responses mediated by multiple types of activated cells. Lack of, or reduced number of Treg cells, or their functional deficit, leads to aggressive lymphoproliferative autoimmune disease in human and mice, as exemplified by IPEX syndrome and *scurfy* phenotype, respectively. Conversely, increasing the number of Treg cells by adoptive transfer (19) or treatment with low-dose [review by Klatzmann and Abbas (51)] or attenuated IL-2 (13–16) reduces inflammation and improves disease. However, increasing the number of Treg cells may not be sufficient, based on the examples where the presence of (or even elevated numbers of) Treg cells does not result in reduced inflammation (52, 53). Without being mutually exclusive, several explanations for this

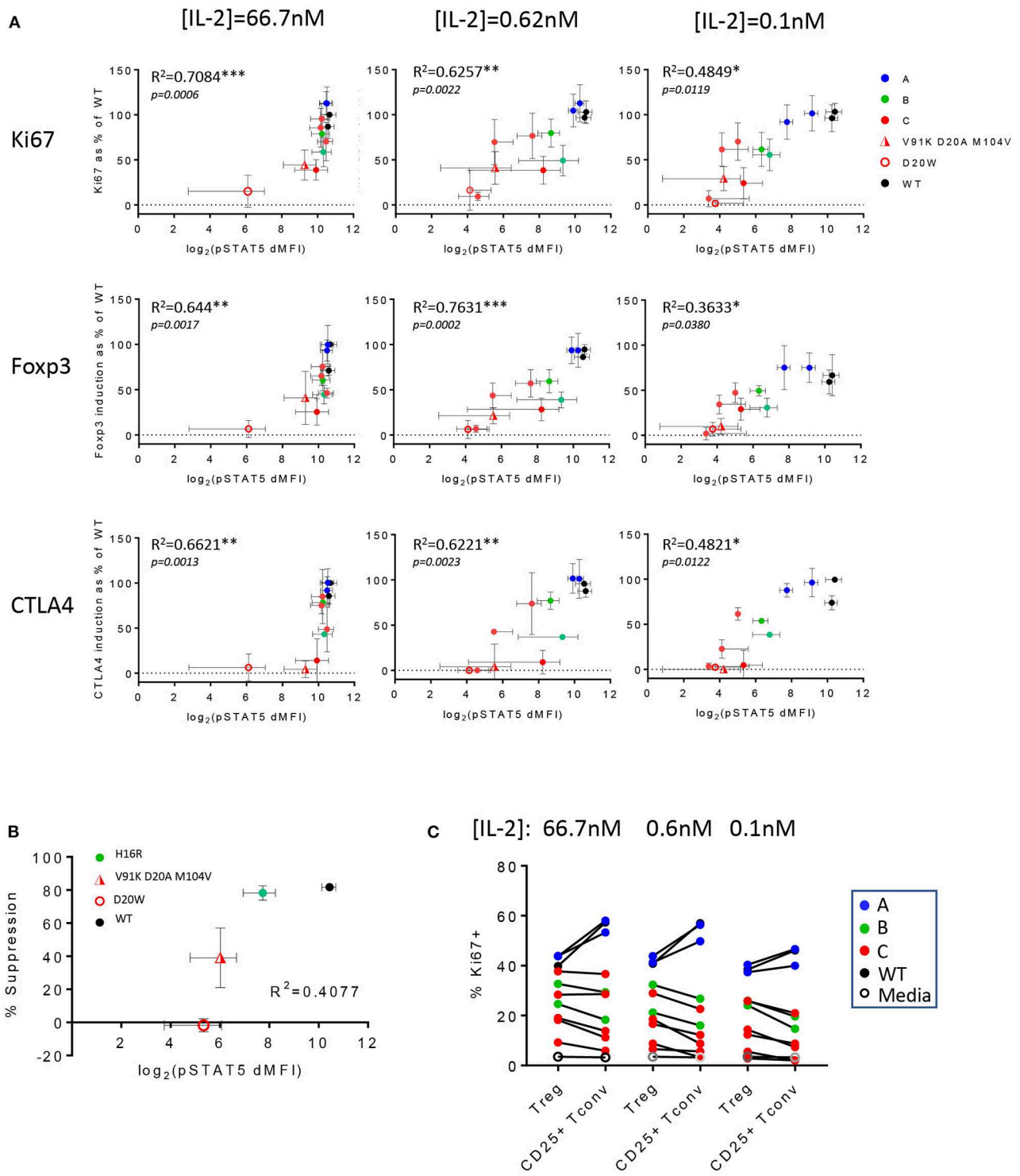

FIGURE 6 | Treg cell functional responses are variably sensitive to IL-2 activity. **(A)** *In vitro* Treg cell proliferation (percent Ki67 positive of Treg cells) and Foxp3 and CTLA4 expression (MFI of Foxp3+ or CTLA4+ cells in Treg population) at IL-2 concentrations 66.7, 0.62, and 0.1 nM were converted to % response relative to wildtype IL-2 at 66.7 nM as the reference and plotted against pSTAT5 MFI (in log2 scale) values that were generated from the whole blood cell assay. The data are combined results from three different donors. The error bars indicate standard deviation. R^2 values were calculated from Pearson *r* generated by correlation analysis, and the corresponding *p*-values are shown. Data points are color-coded by class, except the two weakest muteins (V91K D20A M104V and D20W). **(B)** Correlation plot of *in vitro* suppression and pSTAT5 signal in Treg cells. The y-axis represents the percent suppression of CD8 T cell activation read out by inhibition of activation marker expression in an 1-day Treg:CD8 T coculture assay at [IL-2] = 66.7 nM and Treg:CD8 T ratio of 1:1. One class B mutein (H16R) and the two weaker class C

(Continued)

FIGURE 6 | muteins (V91K D20A M104V and D20W) were evaluated against wildtype IL-2. The x-axis represents the pSTAT5 MFI data from the whole blood cell assay shown in log2 scale. The percent suppression represents the average ± SD values from two donors. The R^2 value from the correlation analysis is shown. **(C)** Wildtype IL-2 and the muteins' activities on Treg vs. CD25+ Tconv are summarized in a pairwise comparison using proliferation as readout (indicated by % Ki67 positive) at three different IL-2 concentrations. The data points are color-coded according to the mutein class.

gap may be considered. One is that wildtype IL-2 induces a much more potent response in inflammatory non-Treg cells such that it undermines any enhancement of Treg cells, even at low doses where the Treg:CD25+ Tconv selectivity is increased. Second possibility is that the IL-2-mediated effects on Treg cells are limited in active disease due to the presence of pro-inflammatory cytokines that have been shown to destabilize Treg cell phenotype and function (54–56) or because a gap in antigen specificity of IL-2-stimulated Treg cells confounds the targeting of these cells to appropriate tissue(s). Another explanation may be that the amount of IL-2 signal required to enhance the functional fitness of Treg cells is higher than that for inducing proliferation and/or to protect from apoptosis. In this scenario, an attenuated IL-2 signal such as that generated by low-dose IL-2 treatment may increase the number of circulating Treg cells with enhanced expression of the various Treg cell phenotypic markers, but it may not be sufficient to enhance their suppressor function. In all these scenarios, the ultimate effects of IL-2 stimulation is likely additionally influenced by synergistic contribution of signals mediated by T cell receptor, TGFβ and/or members of the TNF receptor family.

Our panel of engineered IL-2 muteins induced a wide range of pSTAT5 signal through the IL-2 receptor. Analysis of these muteins whose maximal activity is attenuated compared to wildtype IL-2 allowed us to assess the impact of attenuated signal beyond the limits of the narrow concentration range that normally induces Treg-selective effects. Our data confirm the basic rationale for why the previously reported examples of attenuated IL-2 demonstrate increased selectivity for Treg cells vs. CD25+ Tconv and CD8 T cells. Importantly, our data directly demonstrate that Treg, Tconv, and CD8 T cells possess intrinsically different sensitivity to IL-2 signal. We restricted our comparison to CD25+ gated populations to compare Treg vs. CD25+ Tconv cell responses, however, discrepancies in the relative levels of CD25 expression is likely to be a factor in the observed difference in sensitivity. In this scenario, our observation that class A muteins selectively retain maximal STAT5 response in Treg cells while demonstrating a significant attenuation toward CD25+ Tconv cells at saturated receptor occupancy underscores the contribution of CD25 to overall IL-2 activity in Treg cells, consistent with its known role in facilitating the capture of IL-2 and stabilizing the heterotrimeric receptor. For these muteins, elevated CD25 expression may be sufficient to compensate for the slight loss of affinity to the IL-2RB chain which limits the maximal signal. In this context, it is of relevance that CD25 expression on Treg cells has been reported to be reduced in some diseases (e.g., SLE), which may limit their sensitivity to far-attenuated IL-2 muteins. We also note that despite the attenuated pSTAT5 response, non-Treg cells proliferated disproportionately better than Treg cells in response to wildtype IL-2 and class A muteins *in vitro*. Thus, attenuating the downstream biological responses in these cell types requires much greater attenuation that is indicated by the pSTAT5 readout.

Our analysis showed a strong positive correlation between pSTAT5 signal and multiple downstream biological responses relevant for Treg cell expansion and function. This was not surprising given that much of Treg cell response has been shown to be driven by activated STAT5 (21). Interestingly, the correlation became weaker at lower concentrations, as the receptor occupancy fell below maximal level, and we made two observations. First, Treg cells tolerate a great degree of attenuation of IL-2 signal, indicating that the threshold IL-2 signal required to induced Treg cell response is much lower than previously expected. Second, although the muteins induced Treg cell response that was commensurate with their relative maximal pSTAT5 response, the absolute response did not change significantly over a wide concentration range. It should be noted that in these *in vitro* assays, IL-2 dependent enhancement of Treg cell proliferation requires additional signals, e.g., pre-stimulation via the antigen receptor. Thus, the threshold IL-2 signal indicated in this context is somewhat confined by its requirement for synergistic signals or priming of Treg cells that permit the downstream response. Nonetheless, this is likely relevant for Treg cell responses *in vivo*. Homeostatic maintenance and function of Treg cells require both TCR signal (57, 58) and IL-2 signal (33, 34) and these two signals likely synergize to enhance Treg cell function. Since Treg cells continuously interact with antigen presenting cells *in vivo* that provide TCR and other types of signals (57–61), our results directly show that the availability of IL-2 remains a key factor that controls the Treg cell response *in vivo*, even when it acts in synergy with additional pathways. Using an *in vitro* assay, we demonstrated that the attenuated IL-2 muteins enhanced Treg cell suppressor activity that also correlated with their biochemical activity quantified by the pSTAT5 signal. Surprisingly, V91K D20A M104V, a mutein with a >25-fold reduction in potency, was still able to significantly enhance Treg cell fitness to inhibit activation of effector T cells, even though this same mutein showed hardly any activity in enhancing the expression of Treg cell markers such as Foxp3, CD25, and CTLA4. These results together suggest that, somewhat contrary to our initial hypothesis, a stronger IL-2 activity is required to maintain the high level expression of lineage and activation markers associated with Treg cell stability than to enhance Treg cell proliferation and suppressor function. In human Treg cells, it has been shown that Foxp3 expression is required but not sufficient to maintain Treg cell phenotypic stability and suppressor function [review by Bacchetta et al. (62)]. IL-2 provides a key signal, via STAT5 activation, to induce and maintain Foxp3 transcription. However, IL-2 is likely to influence

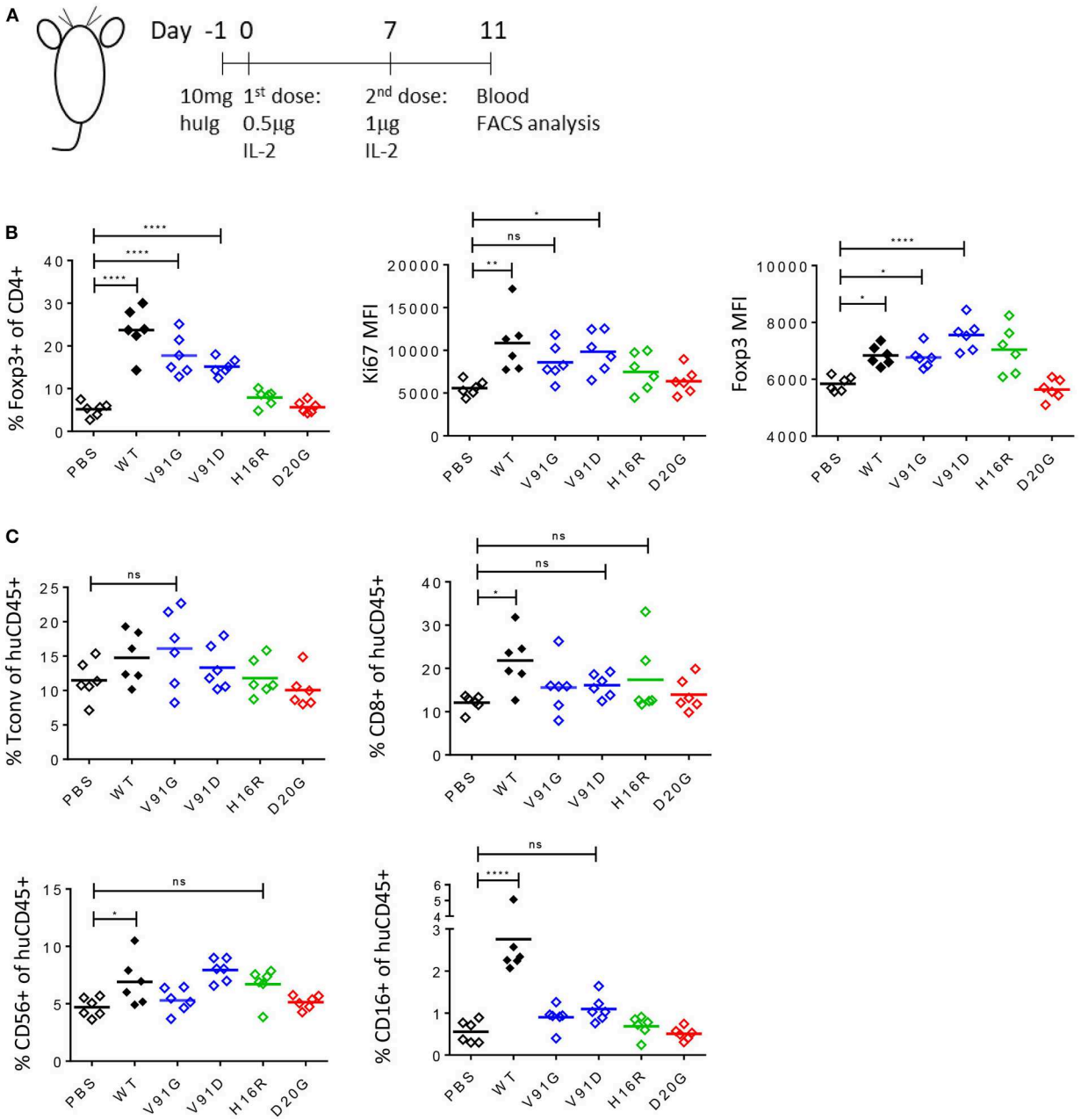

FIGURE 7 | Attenuated IL-2 muteins induce Treg cell expansion with enhanced Treg:NK selectivity *in vivo*. **(A)** Study design schematic. One day before the first dose, humanized NSG mice were treated with 10 mg of human IgG. On day 0, wildtype IL-2 or IL-2 mutein was dosed s.c. at 0.5 µg per mouse, followed by a boost on day 7 at 1 µg per mouse. Blood was analyzed 4 days after the second dose, on day 11. Six animals per group were treated. Shown in the graphs, PBS-treated group served as vehicle control, and the muteins are color coded to represent class A (blue), B (green), and C (red). Ordinary one-way ANOVA analysis was performed to determine statistical significance of the comparisons between the wildtype- or IL-2 mutein-treated group and the PBS-treated group. **(B)** Attenuated IL-2 muteins induce human Treg cell response *in vivo*. Treg cells are gated based on Foxp3 expression and the representation of Treg cells are shown as percent of Foxp3+ in CD4 T cells. The levels of Ki67 and Foxp3 expression on Treg cells are shown as Ki67 MFI and Foxp3 MFI, respectively. $^{*}p < 0.02$; $^{**}p < 0.0025$; $^{****}p < 0.0001$. **(C)** The sizes of the various non-Treg cell compartments post IL-2 treatment are shown as percent of human CD45+ cells; Tconv (Foxp3– CD4 T), CD8 T, and CD56+ CD3– and CD16+ CD3– NK cell data are shown. $^{*}p < 0.04$; $^{****}p < 0.0001$.

additional pathways in Treg cells that are distinct from its effects on Foxp3 expression, as has been suggested previously (63) and in this context is interesting to consider that IL-2 may be able to support pathways required for Treg cell function independently of its effect on the Foxp3 expression. Since our study focused on evaluating the IL-2 mutein activity in mature human Treg cells purified from normal healthy people, our data speaks to the relatively stable functional phenotype of mature Treg cells

that persists in response to attenuated IL-2 signal, even as the *foxp3* transcript level declines. Additional studies are required to address whether *de novo*/*in vivo* activity of IL-2 muteins on Foxp3 expression vs. suppressor activity shows similar disconnect. We also observed that the *foxp3* enhancer region remained highly demethylated in Treg cells treated with weak muteins, even though the *foxp3* transcript and protein levels showed significant reduction in these cells. These results suggest that, in highly purified Treg cells from normal healthy people, IL-2 signal plays a more prominent role in stabilizing *foxp3* transcript than in regulating the methylation status of these enhancer region(s).

From a therapeutic view point, it is difficult to predict the Treg cell number or the level of suppressor function that would sufficiently inhibit pathogenic inflammatory response *in vivo*. However, if the threshold requirements for IL-2 in enhancing or maintaining the Treg cell number, function, and lineage stability are indeed variable, the *in vivo* efficacy of an attenuated IL-2 mutein or low dose IL-2 in disease is more likely to be limited by its inability to maintain the stability of Treg cells, as may be indicated by the expression of Foxp3 and other markers, and by its Treg:CD25+ Tconv selectivity, rather than insufficient activity toward immediately enhancing Treg cells' suppressor function. Addressing this question would require evaluation of these muteins' activities in *in vivo* disease models, which we were not able to perform due to anti-drug antibody (ADA) response to human IL-2 muteins containing human Fc in mice and the difference in the relative potencies of human IL-2 muteins on mouse Treg and CD25+ Tconv cells (data not shown).

IL-2 is required for Treg cell homeostasis and function, and it also acts as a potent driver of Teff and NK cell responses. Wildtype IL-2 is extremely efficient in assembling and productively engaging its receptor, and it exhibits a narrow linear activity range. As a result, the means to control the level of IL-2 signal with wildtype IL-2 is limited. Our study showed that, in designing the next generation of engineered IL-2 as a therapeutic, attenuated IL-2 as a class is superior to wildtype IL-2 in enhancing the Treg:non-Treg selectivity and that a robust activity in increasing Treg cell function and stability are key factors that should be considered in addition to expansion in number. With appropriately attenuated muteins, we anticipate being able to generate a much more controlled Treg-selective IL-2 responses over a considerably wider dose range than with wildtype IL-2 in patients. Additionally, these IL-2 muteins will serve as powerful tools to explore the biology of IL-2 in the many facets of its contribution to immune regulation.

MATERIALS AND METHODS

Human IL-2 Mutein Design and Material Production

Structure based designs were evaluated through analysis of the 2B5I with IL-2 cytokine receptor complex (CD25, IL2RB, IL2RG, and IL-2) using Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). Nucleotide changes were introduced using the Geneious software suite. gblock fragments from IDT-DNA were cloned into a mammalian expression vector using Golden Gate cloning system (ThermoFisher). Constructs encoding effector Functionless Fc fusions (64) of IL-2 variants were stably integrated into CHO K1 cells and expressed at 32°C in a 6-day batch production. The proteins were purified from clarified culture media using MabSelect SuRe affinity column (GE Healthcare) followed by HiTrap Desalting (GE Healthcare), and final purification was performed on a Superdex 200 Increase size exclusion column (GE Healthcare). Proteins were stored at 0.5–3 mg/ml in 10 nM Acetate pH 5.2, 100 M NaCl for biophysical and functional assays.

Human Whole Blood Phospho STAT5 Assay

In vitro STAT5-phosphorylation analysis was performed using human whole blood collected from healthy volunteer with informed consent under Amgen Research Blood Donor program. Following incubation with IL-2 muteins for 30 min at 37°C, samples were treated with pre-warmed Lyse/fix buffer (BD Biosciences) and incubated for further 10 min at 37°C. Fixed and lysed blood sample were subsequently stained for CD25 (2A3, BB515, BD Biosciences), and permeabilized by incubating overnight at −20°C in pre-chilled Perm Buffer III (BD Biosciences). The next day, samples were stained for CD3 (UCHT1, Pe-Cy7, Invitrogen), CD4 (SK3, BUV395, BD Biosciences), CD8 (SK1, BV711, BioLegend), Foxp3 (259D, AF647, BioLegend), and pSTAT5 (47, PE, BD Biosciences). For NK cell panel, fixed and lysed blood samples were subsequently stained using CD25 (2A3, BV421, BD Biosciences), CD56 (HCD56, AF488, BioLegned), and CD16 (3G8, BV510, BioLegend) and then permeabilized by incubating overnight at −20°C in pre-chilled Perm Buffer III (BD Biosciences). The next day, samples were stained for CD3 (UCHT1, Pe-Cy7, Invitrogen), CD4 (BUV395, SK3, BD Biosciences), CD8 (BV711, SK1, BioLegend), Foxp3 (259D, PE, BioLegend), and pSTAT5 (47, AF647, BD Biosciences); data were acquired using BD FACSymphony (BD Biosciences) and analyzed using FlowJo software (v10.6.1, BD).

Biacore Affinity Measurements

For analysis of binding to CD25, a standard SPR binding assay was employed. A CM5 (GE Healthcare) Biacore T100 chip was pre-conditioned according to the manufacturer's suggested protocol. Recombinant CD25 protein (R&D Systems, cat #223-2A/CF) was reconstituted as a 0.1 mg/mL solution in PBS, prior to dilution into 10 mM sodium acetate (pH = 4.5) immobilization buffer (GE Healthcare), for a final CD25 concentration of 1 μg/mL. Approximately 90 RU of ligand was coupled to the sensor surface using standard EDC/NHS coupling protocols provided by the manufacturer. For creation of a reference flow cell, activation and quenching of the sensor surface without ligand attachment was performed. For assay of the analyte panel, all proteins were prepared as 3-fold dilution series from 500 to 6.2 nM in running buffer (PBS + 0.05% Tween-20). Prior to data collection, regeneration scouting was performed, and surface stability of CD25 assessed after repeat injections of 50 nM WT IL2-Fc parent standard. CD25 surface activity post-coupling was estimated to ∼50%. For binding assay, injection times of 60 s (500–55 nM) or 120 s (18–6.2 nM) were

used to achieve steady state binding levels, followed by a 5 min dissociation phase prior to regeneration of the surface using 2 × 15 s pulses of 10 mM glycine*HCL (pH = 1.5). For each concentration series, duplicate injections were performed in sequence. A flow rate of 75 μL/min was used throughout the analysis. Data analysis was performed by double referencing each sensorgram against both reference flow cell and blank injections. Steady state binding (RU) levels were used during a global fitting routine of the entire dataset to a 4-parameter logistic function to derive EC50 values.

For analysis of binding to IL2RB, Biacore Series S, SA chip was pre-conditioned per the manufacturers suggestion prior to immobilization of minimally biotinylated recombinant human IL2RB-huFc (Sino Biological 10696-HO2H) to a density of between 400 and 500 RU. One untreated flow cell was utilized at a reference channel. For screening of the mutein analytes, all were prepared as 1,000 nM solutions in running buffer (PBS-P+, +0.2% BSA, +0.01% sodium azide). Analysis was conducted by injecting analyte for 40 s at flow rate of 30 μL/min, followed by 60 s dissociation. Regeneration of the active surface was achieved by 15 s pulses of 1 M $MgCl_2$ in sodium Acetate (pH 5.5). Surfaces were tested as stable and binding deemed reproducible by repeated injection of the WTIL2-Fc protein control prior to initiation of the screen. Data were double referenced, and the maximum signal at steady state was recorded. The data were reported as % binding to the control, calculated as follows:

$$\% \text{ control} = (\text{mean RU Sample})/(\text{mean RU WTIL2-Fc control}) \times 100$$

The mean of each sample was from duplicate injections; the controls were injected at least 4 times per run. RU was taken at binding maximum, or steady state with 1,000 nM concentration.

Human PBMC Proliferation Assay

Total PBMCs were stimulated with α-CD3 antibody (OKT3, Biolegend) at 0.1 μg/ml for 2 days in complete RPMI 1640 media containing 2% heat-inactivated human AB serum (Sigma-Aldrich), washed, and rested in complete media for 5 days. On day 7, cells were harvested and 200 k cells were cultured in complete media containing only IL-2 mutein at titrating concentrations in a 96-well flat bottom plate. Five days later, cells were harvested and labeled with a viability dye (LIVE/DEAD Fixable Near-IR Dead Cell stain Kit, ThermoFisher Scientific) per manufacturer's protocol and stained for cell surface markers: CD3 (BUV805, BD Biosciences), CD4 (BUV395, BD Biosciences), CD8 (BV510, Biolegend), CD25 (BB515, BD Biosciences), and CTLA4 (a-CD152-PE/Cy7, Biolegend). Stained cells were fixed and permeabilized using the Foxp3 fix/perm buffer kit (eBioscience) and stained for Foxp3 (Alexa Fluor 647, Biolegend) and Ki67 (BV421, BD Biosciences). Samples were acquired on BD FACSymphony (BD Biosciences) and analyzed using FlowJo software.

The raw values of the percent Ki67-positive or Foxp3 and CTLA4 median fluorescence intensity were converted to percent response (% response) based on the values obtained from cells stimulated with wildtype IL-2 at 66.7 nM or media only (baseline). The equation that was used for conversion is as follows:

$$\% \text{ response} = (\text{experimental} - \text{baseline})/(\text{value at wildtype IL-2 @ 66.7nM} - \text{baseline}) \times 100$$

Human Treg Cell Purification and Stimulation

PBMCs isolated from healthy human donor leukopaks (AllCells) by density gradient centrifugation (Ficoll Paque Premium, GE-Healthcare) were used for isolating CD4+ CD127Low/-CD25+ Treg cells. CD4+ CD127Low/- CD25+ Treg cells were isolated from PBMC using EasySep human CD4+ CD127Low CD25+ Regulatory T cell isolation kit (STEMCELL Technologies). Isolated Tregs were cultured in tissue culture plate pre-coated with anti-CD3 at 2 μg/ml (OKT3, BioLegend) plus WTIL2-Fc, H16R, V91K D20A M104V, or D20W IL-2 muteins at 66.6 nM in RPMI1640 media containing 10% Heat-inactivated Human AB serum (Sigma) and supplemented with GlutaMAX, 1mM Sodium Pyruvate, Non-essential Amino Acids, 10 mM HEPES, 100 U/ml Penicillin and Streptomycin, β-Mercaptoethanol (all from GIBCO, Life Technologies) for 3 days at 37°C.

In vitro Treg Suppression Assay

To evaluate the activity of IL-2-treated Treg cells, purified CD8+ T cells were used as responder cells in a Treg:CD8 T coculture system. CD8+ T cells were purified from PBMCs isolated from healthy human donor derived leukopaks (AllCells) using EasySep CD8+ T cell Negative selection kit (STEMCELL Technologies) and were cultured with purified and pre-stimulated (as described in the human Treg cell purification and stimulation section) human Treg cells at varying ratios, in the presence of anti-CD3/anti-CD28 magnetic DynaBeads (Invitrogen) added at 1:12 bead:T cell ratio. Suppression was measured by inhibition of activation marker CD25 expression on responder CD8+ T cells after overnight incubation at 37°C.

Percent suppression was calculated using changes in CD25 MFI as a measure of activation in responder CD8 T cells using the following formula:

$$\% \text{ Suppression} = 100 - [(\text{MFI in the presence of Treg} - \text{MFI in non-activated Responder only})/(\text{MFI in activated Responder only} - \text{MFI in non-activated Responder only}) \times 100].$$

Statistical data analysis was performed using GraphPad Prism software (v7.04, GraphPad Software, Inc.). To compare the effect of different IL-2 muteins and WTIL2-Fc in Treg suppression assay, 2-way ANOVA test was used with Tukey *post-hoc* test.

Taqman Analysis of STAT5-Inducible Genes

Total RNA was extracted from Treg cells stimulated under indicated conditions using Trizol reagent (Invitrogen) according

to the manufacturer's protocol. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen). Quantitative PCR was performed using FAM-MGB labeled Taqman probes purchased from Invitrogen.

Foxp3 Bisulfite Amplicon-Seq DNA Library Preparation and MiSeq Analysis of IL-2 Mutein Treated Treg Cells

CD4+ CD127low/- CD25+ Tregs were isolated from PBMCs from male donors using EasySep CD4+ CD127low/- CD25+ Regulatory T cell isolation kit as previously mentioned. Enriched Tregs were cultured in complete RPMI media containing 10% heat-inactivated human AB serum and WTIL2-Fc, H16R, V91K D20A M104V, or D20W at 66.6 nM for 36 h at 37°C. CD25– Tconv cells were also isolated from the unbound fraction of the Treg purification process and further enriched by CD25 bead depletion (Miltenyi) and cultured without any stimulation as a negative control.

Genomic DNAs were extracted using Gentra Puregene Cell Kit (Qiagen) protocol. Fifty nanogram gDNA was used for the bisulfite conversion following the protocol of EpiTect Bisulfite Kit (Qiagen). Five nanogram converted gDNA was amplified by PCR, and amplicons were purified using SPRIselect beads (Beckman Coulter) for next generation sequencing.

To prepare the Amplicon-Seq DNA library, we processed ∼50 ng PCR products of each Bisulfite treated sample using the Nextera XT (Illumina: FC-131-1096) kit per manufacturer's suggested protocols for DNA library preparation. All samples were multiplexed using Illumina-supplied Index 1 and Index 2 primers followed by a 12-cycle PCR reaction. The DNA libraries were then cleaned up using the AMPure XP beads. Agilent TapeStation was used to perform quality control (QC) on PCR amplified libraries. All libraries were sized between 200 and 550 bp. Four nanometer amplified library of each sample was then pooled together from the DNA library and sequenced using the 300-cycle (MiSeq Reagent Kit v2) sequencing kit format. The paired-end sequencing was performed on the Illumina MiSeq and the results were analyzed on ArrayStudio.

In vivo Activity Assay

Female humanized NSG (NOD scid gamma) mice (NSG mice reconstituted with human CD34 stem cells, JAX Laboratory) were randomized based on the percentage of human CD45 cell engraftment. Ten milligrams of human gamma globulin were injected into each mouse subcutaneously 1 day before (d −1) IL-2 mutein dosing to block non-specific binding to unoccupied human FcRs. Mice were dosed subcutaneously with 0.5 μg of various IL-2 muteins or control on day 0 and a second boost dose was given at 1.0 μg per mouse on day 7. Our choice for weekly dosing and analysis on day 4 post the boost was based on the prolonged PK/PD effects reported for a similar molecule in non-human primate (NHP) and nod.scid mice (65). Non-terminal retro-orbital bleeding was performed and 100 μL of whole blood was obtained from each mouse on day 11 (day 4 post boost) for FACS staining. Whole blood was stained with cell surface markers: CD3 (FITC, BD Biosciences), CD56 (PerCP, BD Biosciences), CD16 (PE-Cy7, BD Biosciences), CD25 (APC, Miltenyi), CD45 (APC-Cy, BD Biosciences), CD4 (V500, BD Biosciences) for 20 min before red blood cells were lysed in BD FACS lysing solution (BD Biosciences). Cells were then fixed and permeabilized, and stained for Foxp3 (PE, Biolegend) and Ki-67 (V450, BD Biosciences) using Foxp3 staining buffer set (eBioscience). Data were acquired on LSRII (BD Biosciences) and analyzed by Flowjo. For statistical analysis, ordinary one-way ANOVA analysis was performed using GraphPad Prism software (v7.04, GraphPad Software, Inc.).

All experimental studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Amgen. Animals were housed at Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities (at Amgen) in ventilated micro-isolator housing on corncob bedding. Animals had access *ad libitum* to sterile pelleted food and reverse osmosis purified water and were maintained on a 12:12 h light:dark cycle with access to environmental enrichment opportunities.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Amgen.

AUTHOR CONTRIBUTIONS

AG performed majority of the experiments. DB designed and produced IL-2 muteins. KC performed the CD25 and IL2RB affinity measurements. M-ZW, JL, and C-ML performed the TSDR assay. Y-LH performed the *in vivo* studies with the humanized NSG mice. AC performed the Taqman analysis. SS conceptualized, performed experiments, and wrote the manuscript. All authors wrote the methods and figure legends for the data they generated.

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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.2020.01106/full#supplementary-material

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REVIEW
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Regulation of Human Innate Lymphoid Cells in the Context of Mucosal Inflammation

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Since their identification as a unique cell population, innate lymphoid cells (ILCs) have revolutionized our understanding of immune responses, leaving their impact on multiple inflammatory and fibrotic pathologies without doubt. Thus, a tightly controlled regulation of local ILC numbers and their activity is of crucial importance. Even though this has been extensively studied in murine ILCs in the last few years, our knowledge of human ILCs is still lagging behind. Our review article will therefore summarize recent insights into the function of human ILCs and will particularly focus on their regulation under inflammatory conditions. The quality and intensity of ILC involvement into local immune responses at mucosal sites of the human body can potentially be modulated via three different axes: (1) activation of tissue-resident mature ILCs, (2) plasticity and local transdifferentiation of specific ILC subsets, and (3) tissue migration and accumulation of peripheral ILCs. Despite a still ongoing scientific effort in this field, already existing data on the fate of human ILCs under different pathologic conditions clearly indicate that all three of these mechanisms are of relevance for the clinical course of chronic inflammatory and autoimmune diseases and might likewise provide new target structures for future therapeutic strategies.

Keywords: innate lymphoid cells, mucosal inflammation, human immune system, cytokine, ILC plasticity, tissue migration

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INTRODUCTION

Having been overlooked for ages, helper innate lymphoid cells (ILCs) have been increasingly recognized as key immunological players since their discovery as a distinct cell population in 2010 (1–3). Since then, as a result of an immense amount of scientific effort, a prominent role has been assigned to ILCs as initiators and amplifiers of protective but also detrimental immune responses in various tissues, making them interesting potential therapeutic targets (4, 5).

Phenotypically, ILCs are classified as lymphoid cells that lack the expression of lineage markers defining any known lymphoid or myeloid cell population (6). Functionally, ILCs share core effector features with T cells, even though they are characterized by a lack of rearranged antigen-specific receptor expression. This enables full activation of ILCs independent from the antigen-presentation and -recognition machinery and thereby the induction of rapid immune responses (7). After their stepwise development from a common lymphoid progenitor cell [reviewed elsewhere (8–10)], mature ILCs can be categorized into three main subgroups by analogy to T cells, based on their dependency on transcription factors and the secretion of effector cytokines: type-1, type-2, and type-3 ILCs (ILC1s, ILC2s, and ILC3s, respectively) (11). ILC1s can be further subdivided into

cytotoxic NK cells and helper ILC1s (12). Whereas, classic NK cells are well-known to mediate a potent cytolytic effector function and have been extensively studied and reviewed already (13, 14), this review will focus on helper ILCs in particular. While helper ILC1s critically depend on the transcription factor T-bet and are able to amplify immune responses against intracellular pathogens via an extensive release of IFN-γ and TNF-α (15, 16), ILC2 function is regulated by GATA-3 and RORα as key transcription factors, and the effector cytokines IL-5, IL-13, IL-9, and IL-4 relevantly impact the resolution of helminth infections (6, 17, 18). Finally, analogous to type-17 T helper (Th17) cells, RORγt represents the master transcription factor of ILC3s, including lymphoid tissue inducer (LTi) cells as well as non-LTi ILC3 subsets. While LTi cells play a particular role in lymphoid organogenesis, ILC3s in general are characterized by the secretion of IL-17A, IL-22, and GM-CSF and are thereby involved in the immunological control of extracellular microbes (19–21). In addition to these three classical subgroups, in analogy to regulatory T cells (Tregs), regulatory ILCs (ILCregs) were recently identified in the intestine that suppressed ILC1s and ILC3s in an IL-10-dependent manner, while TGF-β served as autocrine growth factor (22).

Helper ILCs are primarily located in close proximity to mucosal barriers, like the pulmonary (23) and intestinal epithelium (19), which are highly prone to environmentally driven tissue damage and pathogen entry. There, ILCs are involved in the first line of immune response via the instant release of extraordinary amounts of effector cytokines that orchestrate further immune reactions (24, 25). However, tight control of local ILC numbers and their activation status is crucial to guarantee barrier integrity and tissue homeostasis without the induction of overwhelming and chronic immune responses.

Based on their overall low frequency and redundant functions with T helper (Th) cells as well as the finding that ILC deficiencies appeared to be asymptomatic in humans with competent adaptive immune cells, ILCs were suggested to be expandable under natural conditions. This assumption, however, was only based on a small cohort living under modern hygiene and medical standards (26) and does not seem to hold true under pathological conditions. In severe liver fibrosis, for example, local ILC2 frequencies were exclusively increased while the proportion of Th2 cells was unaltered (27), indicating a particular role for ILCs during fibrotic tissue remodeling. In line with this, a cell-specific regulation and thus activation profile of ILCs and Th cells has been described. ILC2s, for instance, rely on DR3 and IL-9R signaling for activity and survival, which was not the case in Th cells (28, 29). On a functional level, it was particularly the $CD3^-$ lamina propria mononuclear cell (LPMC) fraction that showed significantly increased IL-22 production in inflammatory bowel disease (IBD) patients compared to controls but not Th cells (30), assigning ILCs a distinct and important effector role in disease. Moreover, the rapid availability of effector cytokines and the finding that ILC2s are more potent in the production of IL-5 and IL-13 than are $CD4^+$ T cells in blood and sputum of patients suffering from severe asthma (31) distinguishes ILCs from Th cells, making them a functionally unique cell population. Importantly, ILC activity has been shown to be crucial for efficient T cell responses under various conditions (32–34), demonstrating their far-reaching influence on efficient immunity.

And while ILCs have been shown to be involved in many different immunological phenomena, including host protection, wound healing, anti-tumor immune responses, autoimmunity, graft-vs.-host reaction, chronic inflammation, and fibrosis in numerous murine studies (35–41), the transfer of these findings into the human system and a related functional characterization of ILCs in the context of human disease still remains incomplete. Even though murine and human ILCs share basic characteristics, human ILCs have been shown to markedly differ in several key aspects from their murine counterparts (8, 42, 43), making translational research on human ILCs inevitable. The first important hints of the existence of species-specific ILC biology arose from studies that described variances in the ILC surface marker profile between mice and men. Regarding ILC1s, a distinct subset restricted to an intraepithelial localization and producing IFN-γ in response to IL-12 and IL-15 was described that differs in its αE integrin and NKp44 expression between mice and humans (44, 45). Similarly, two distinct functional subtypes of ILC2s, namely inflammatory and natural ILC2s, could be identified in both species but differed in their surface maker profiles. While, in mice, these subtypes were distinguished by ST2 and KLRG1 expression (46), functionally similar subtypes in humans were rather discriminated by their c-Kit expression (47). In the group of ILC3s, two subtypes secreting mainly IL-22 or IL-17 have been described in varying proportions and with altered marker expression in the two species (45, 48). These phenotypical and numerical differences strongly imply that the localization and activation of murine and human ILCs might also be partly regulated by separate molecular mechanisms. And indeed, on a functional level, there is an ongoing and controversial discussion as to whether ILCs of the two species follow the same mechanistic concept of tissue distribution and maturation in adulthood. While parabiosis experiments in mice strongly suggested that ILCs have a tissue-resident, long-lived nature and mostly excluded their recirculation and organ redistribution upon acute inflammation (49, 50), a very recent study postulated a concept of circulating uni- and pluripotent human ILC precursors that are able to migrate into tissue and undergo final differentiation in response to local environmental signals (51). This permanent presence of ILC precursors in the peripheral blood together with the idea of tissue ILC differentiation (51) is in accordance with the well-described phenomenon of a significant organ accumulation of defined ILC subsets in the context of inflammatory tissue injury. Indeed, patients suffering from IBD show distinct numerical alterations in the ILC composition in the intestinal mucosa that depend on disease duration (15, 52, 53). Moreover, atopic dermatitis, hepatic fibrosis, and chronic rhinosinusitis are associated with an accumulation of ILC2s in skin, liver, and sinonasal tissue, respectively (23, 27, 54). This association strengthens the clinical urgency of directly analyzing human ILCs, especially since most murine studies are biased by the use of specific-pathogen-free or immunodeficient mice without a functional adaptive immune system that do not sufficiently represent the human situation and

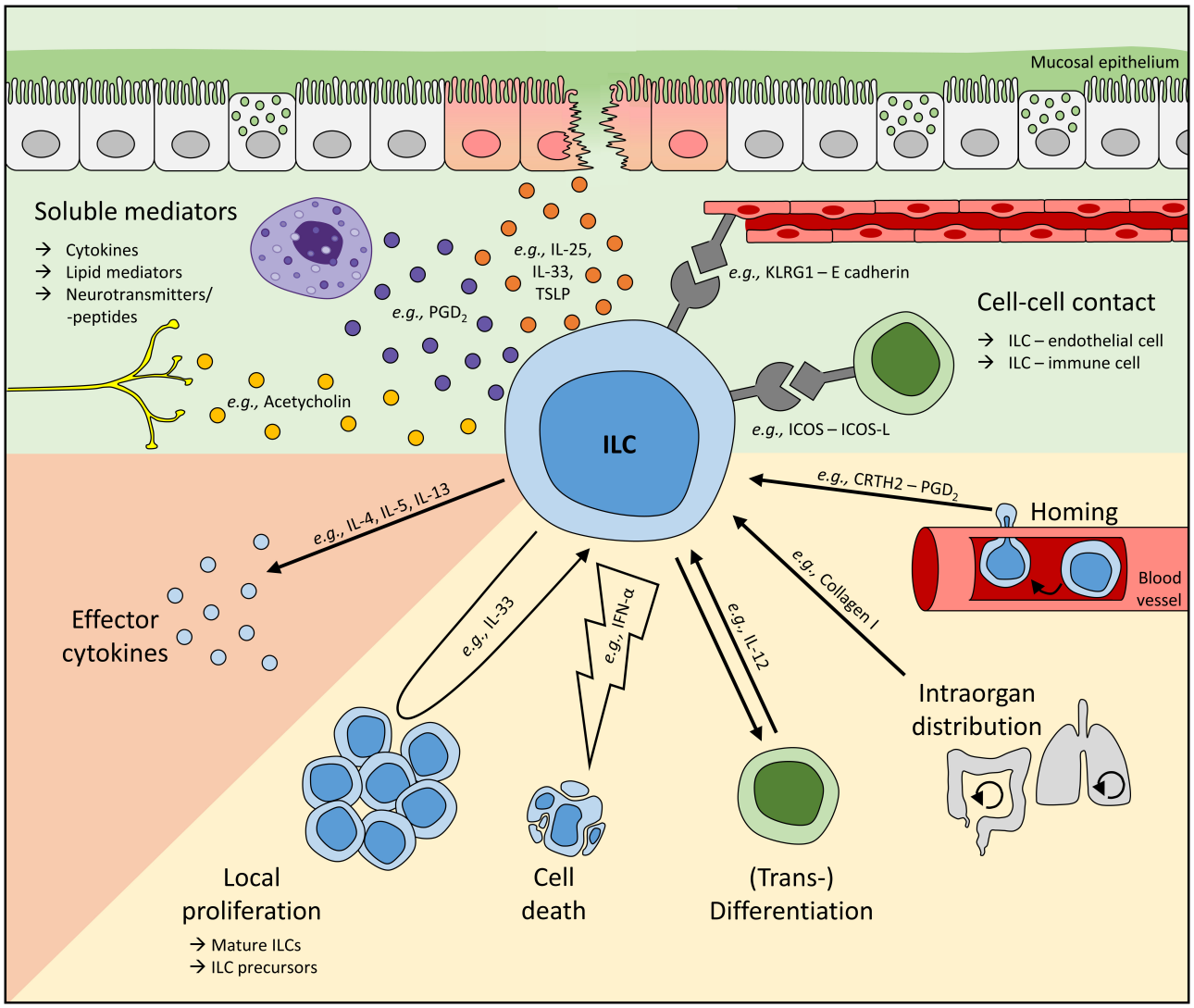

FIGURE 1 | Mechanisms regulating local ILC activity. To adapt ILC functions at mucosal barriers to the respective environmental need, local ILC numbers and their activity can be regulated by soluble mediators or direct cell-cell interactions (green background). To date, cytokines and lipid mediators represent the most commonly described soluble regulators of human ILCs and can be released, for instance, by stress-sensing epithelial cells or other immune cells like mast cells. Furthermore, neurotransmitters and neuropeptides have also been suggested to directly interact with ILCs. In addition, cell-cell contact-dependent regulation of ILCs is based on their interaction with endothelial, stromal, and other immune cells. Upon sensing these signals, ILC activity (orange background) and their local number (yellow background) can be controlled. Tissue ILC counts can be modulated directly by cell death and proliferation or the differentiation of local ILC precursors into mature cells. Moreover, ILCs are plastic cells, enabling the transdifferentiation of one subset into another. Altered local ILC numbers can additionally result from the migration of ILCs either within an organ or from/to a distal site. Representatively, regulators modulating ILC activities on various levels are shown. *In vivo*, multiple mechanisms controlling local ILC activity are likely to act synergistically, enabling the activation or suppression of ILC activities in a highly controlled fashion. However, dysbalanced and overwhelming ILC responses are often unable to successfully fight pathogens or can even trigger inflammatory diseases.

might therefore not allow results to be directly translated into the human system (6).

The following review article will therefore summarize recent insights into the function of human helper ILCs and will focus on their regulation at mucosal sites under inflammatory conditions in particular. The quality and intensity of ILC-driven local immune responses at mucosal tissues can be modulated via the activation status as well as a numerical regulation of local ILCs (**Figure 1**). This potentially involves three different axes: (1) activation or inhibition of tissue-resident ILCs, (2) numerical regulation of mature local ILCs via cell death, proliferation, or differentiation from local precursors or other ILC subsets, and (3) tissue-specific migration and regional accumulation of peripheral ILCs (12, 15, 35, 51, 55). Despite ongoing scientific efforts in this field, already existing data on the fate of human ILCs under various pathological conditions clearly indicate that all three mechanisms relevantly impact the clinical course of chronic inflammatory and autoimmune

diseases and might therefore provide new target structures for future therapeutic strategies.

REGULATION OF HUMAN ILCs

Helper ILCs as Guardians at Mucosal Barriers

Forming large surfaces with the body's outer environment, mucosal tissues, including the respiratory, gastrointestinal (GI), and urogenital tract, have to guarantee stable protection against invading pathogens and various harmful substances. Therefore, the maintenance of epithelial integrity as a physical barrier and the capacity to initiate immediate but controlled mucosal immune responses are essential. Based on their instant, antigen-independent ability to secrete effector cytokines, ILCs represent ideal guardians in mucosal tissues. In line with this, ILCs have been shown to preferentially accumulate in organs with mucosal barriers in close proximity to the epithelium (56, 57).

From the esophagus to the colon, all helper ILC subsets have been described in the human GI tract, with the highest frequencies of total helper ILCs residing in the intestine (58). While ILC1s appeared to be enriched in the human gingivae (59) and esophagus (58), NKp44/NCR$^+$ ILC3s represent the most abundant subtype in the gut (53, 55, 58), suggesting an important function for ILC3s in intestinal homeostasis. In contrast, only low frequencies of ILC2s have been detected in both the upper and lower GI tract (53, 55, 58, 60). Under chronic inflammatory conditions, the local ILC composition is drastically altered in inflamed areas (53), as shown by the distribution of intestinal ILCs in IBD patients (15, 30, 52, 53). Indeed, altered numbers of colonic NKp44$^+$ ILC3s have been described already in early in IBD (15), and IL-22 production by ileal ILCs was shown to be increased in patients with mild or moderate CD (30). In accordance with the common concept that the immunopathogenesis of CD and UC is dominated by type-1 and type-2 immunity, respectively (61), CD patients were also characterized by increased ILC1 frequencies (15, 53) as well as IL-17-secreting ILCs (52), whereas UC patients displayed increased proportions of ILC2s during the course of disease (53). Interestingly, ILCregs were described in the murine and human gut as well, likely serving as a control mechanism to suppress exaggerated immune responses (22). Overall, these disease-dependent alterations of ILC frequencies in the human intestine suggest defined functions of ILC subgroups under specific inflammatory conditions and at different anatomical sites, implicating a milieu-dependent fine-tuning of each subset.

In the respiratory tract, research has focused on the ILC2 subset in particular, given the pivotal role of type-2 mediated immunity in allergic airway diseases (62). Nevertheless, all three helper ILC subsets have been described in lung tissue, with ILC2s and ILC3s being most abundant (55, 60, 63). During adulthood, several disorders associated with acute and chronic inflammation of the lung are characterized by altered ILC frequencies. For instance, asthmatic patients showed increased ILC2 frequencies and effector cytokines in peripheral blood, sputum, and bronchoalveolar lavage (BAL), which turned out to correlate with the severity of clinical symptoms (31, 64–68). Next to this allergic context, lung inflammation resulting from infection with *Mycobacterium tuberculosis* was characterized by reduced blood pools of albeit activated ILC1s, ILC2s, and ILC3s and a corresponding accumulation of these cells in the infected lung tissue (69). The observations that ILC2s were enriched in the BAL of patients with idiopathic pulmonary fibrosis (37) and that the destructed lung tissue of patients with chronic obstructive pulmonary disease (COPD) showed elevated local ILC1 and NKp44$^-$ ILC3 frequencies at the expense of ILC2s (55) point to a potential reciprocal interference between pulmonary ILCs and fibrotic tissue remodeling. Furthermore, ILC2s are present in nasal tissue, where they also showed increased proportions upon upper airway inflammation, such as for example, in patients suffering from allergic rhinitis (70, 71) and chronic rhinosinusitis with nasal polyps (55, 60, 72). Contrarily, nasal polyps in the context of cystic fibrosis were dominated by enhanced percentages of NKp44$^-$ ILC3s (72). These findings indicate that various helper ILC subsets play a key role in inherited as well as allergen-, bacterial-, and environmental-driven inflammatory lung disorders. Nevertheless, inconsistent study designs and patient and control cohorts, as well as variable marker combinations defining ILC subsets, led to partly controversial results (64, 70, 71) and impede larger meta-analyses. Based on the current pandemic situation induced by the new coronavirus, SARS-CoV-2, the question of an ILC involvement in the resulting lung disease, COVID-19, is being raised. Indeed, there are good grounds for speculating about a relevant disease-modulating capacity of mucosal ILCs in this viral infection: ILCs are present in the lung tissue even under steady-state conditions (55, 60, 63) and are located in direct proximity to the respiratory epithelium (57) and thus to ACE2-expressing pneumocytes, which have been described as the predominant entry and replication site of SARS-CoV-2 (73). Accordingly, diffuse alveolar damage, as detected histologically in lung biopsies of COVID-19 patients (74), represents a well-described trigger of local ILC activation, classically resulting in the initiation and regulation of far-reaching immune responses (75). Besides epithelial cell-derived alarmins, the activation status of ILCs could also be influenced by immune cell-secreted cytokines upregulated in the course of severe COVID-19 (76, 77), such as IL-6 (stimulatory effect on human ILC3s) or IL-10 (inhibitory effect on ILC2s) (see also **Table 1**). Thus, on a functional level, a relevant contribution of activated pulmonary ILCs to the anti-viral immune response and to the consolidation of epithelial damage can be expected and might mainly be relayed via an excessive release of ILC-derived cytokines. And indeed, altered NK cell frequencies in COVID-19 patients (109) have been the first proof that infection with SARS-CoV-2 does modulate the ILC compartment. Especially in severe COVID-19 cases, NK cell percentages turned out to be downregulated in line with the overall observed lymphocytopenia (109, 110). However, upon recovery, restoration of NK cell frequencies has been described (109, 110), implicating a relevant function for NK cells in the resolution of this viral infection. In general, NK cells, together with helper ILC1s, are considered to be important effector cells, fighting various viral diseases and representing an

TABLE 1 | Local regulators of ILC activity.

ILC subgroup	Regulator	Class	Regulation	ILC origin	Pathophysiological context	References
ILC1	IL-12	Cytokine	+	Tonsils, peripheral blood	Immunity to mycobacteria, CD	(15, 44, 55, 78)
ILC1	IL-15	Cytokine	+	Tonsils	CD	(44)
ILC1	IL-18	Cytokine	+	Intestine	Commensal and pathogenic gut microbiota	(79)
ILC1	IL-1β	Cytokine	+	Intestine	Commensal and pathogenic gut microbiota	(79)
ILC1	TGF-β	Cytokine	–	Peripheral blood	IBD	(80)
ILC2	IL-33	Cytokine	+	Peripheral blood; nasal polyps, fetal gut, tonsils	IBD, asthma	(18, 55, 60, 68, 81)
ILC2	IL-25	Cytokine	+	Peripheral blood; nasal polyps, fetal gut, tonsils	IBD, asthma	(18, 60, 68, 81)
ILC2	TSLP	Cytokine	+	Peripheral blood; nasal polyps, fetal gut, tonsils	Chronic rhinosinusitis with nasal polyps, IBD, asthma	(18, 55, 60, 81)
ILC2	IL-1α/β	Cytokine	+	Peripheral blood, tonsils	IBD, COPD	(55, 60, 82)
ILC2	IL-18	Cytokine	+	Peripheral blood	Inflammatory cutaneous diseases	(12)
ILC2	TL1A	Cytokine	+	Peripheral blood	Helminth infection, type-2 lung inflammation	(28)
ILC2	IL-4	Cytokine	+	Peripheral blood	Chronic rhinosinusitis with nasal polyps	(55)
ILC2	IL-10	Cytokine	–	Peripheral blood, nasal polyps	Grass polen immunotherapy	(80, 83)
ILC2	TGF-β	Cytokine	–	Peripheral blood, nasal polyps	Grass polen immunotherapy	(83)
ILC2	IFN-α	Cytokine	–	Peripheral blood	Suppression of airway inflammation	(84)
ILC2	IFN-β	Cytokine	–	Cord blood	Asthma	(85)
ILC2	CCL1	Cytokine	+	Peripheral blood	Anti-helminth and - parasitic immunity	(86)
ILC2	PGD_2	Lipid mediator	+	Skin, peripheral blood	Allergy	(87, 88)
ILC2	LTE_4	Lipid mediator	+	Peripheral blood	Atopic dermatitis	(89)
ILC2	PGI_2	Lipid mediator	–	Peripheral blood	Allergen-induced lung inflammation	(90)
ILC2	PGE_2	Lipid mediator	–	Peripheral blood, tonsils	Allergic lung inflammation	(91)
ILC2	Lipoxin A4	Lipid mediator	–	Peripheral blood	Asthma	(88)
ILC2	Retinoic acid	Vitamin	+	Peripheral blood	Allergic inflammation	(92)
ILC2	1,25D	Vitamin	–	Peripheral blood	Allergic inflammation	(92)
ILC2	ICAM-1–LFA-1	ILC2–immune cell interaction	+	Peripheral blood	IL-33-induced lung inflammation	(93)
ILC2	GITR–GITR-L	ILC2—immune cell interaction	+	Peripheral blood	Allergic lung inflammation	(94)
ILC2	RANK-RANK-L	ILC2–immune cell interaction	+	Peripheral blood, nasal polyps	Chronic rhinosinusitis with nasal polyps	(95)
ILC2	ICOS–ICOS-L	ILC2 –ILC2	+	Peripheral blood	IL-33-induced airway hyperreactivity	(96)
ILC2	ICOS–ICOS-L	ILC2 –iTreg	–	Peripheral blood	Resolution of airway inflammation	(97)
ILC2	MHCII–TCR	ILC2–Th cell	+	Peripheral blood	Helminth infection	(34)
ILC2	NKp30—B7-H3	ILC2–keratinocytes	+	Peripheral blood	Atopic dermatitis	(98)

(Continued)

TABLE 1 | Continued

ILC subgroup	Regulator	Class	Regulation	ILC origin	Pathophysiological context	References
ILC2	KLRG1–E cadherin	ILC2–endothelial cell	–	Skin	Atopic dermatitis	(54)
ILC2	PD-1	Checkpoint inhibitor	–	Peripheral blood	Helminth infection	(99)
ILC3	IL-23	Cytokine	+	Intestine	IBD	(30, 52)
ILC3	IL-1β	Cytokine	+	Intestine	IBD	(30)
ILC3	IL-18	Cytokine	+	Tonsils	Maintenance of tissue integrity	(100)
ILC3	IL-15	Cytokine	+	Tonsils	Maintenance of tissue integrity	(100)
ILC3	IL-6	Cytokine	+	Colon	IBD	(101)
ILC3	TL1A	Cytokine	+	Intestine, tonsils, hematopoietic stem cell-derived	IBD	(30, 102)
ILC3	IFN-α	Cytokine	–	Tonsils	N/A	(100)
ILC3	IFN-γ	Cytokine	–	Tonsils	N/A	(100)
ILC3	TLR2 ligand	Bacterial metabolite	+	Tonsils	N/A	(103)
ILC3	AHR receptor	Bacterial metabolite	+	Tonsils, intestine	*C. rodentium* infection	(104, 105)
ILC3	Bacillus anthracis toxin	Bacterial metabolite	–	Tonsils	Anthrax	(106)
ILC3	Acetylcholin	Neurotransmitter	+	Peripheral blood	Resolution of *E. coli* infection	(107)
ILC3	1,25D	Vitamin	+/–	Tonsils, intestine	IBD	(108)

CD, Crohn's disease; IBD, Inflammatory bowel disease; COPD, Chronic obstructive pulmonary disease.

early source of IFN-γ and TNF-α (111, 112), with the latter being highly upregulated in the plasma of COVID-19 patients (113). Moreover, data acquired in the murine system indicated that pulmonary ILC2s promoted IgM production in B cells and thus supported early humoral immunity directed against respiratory antigens (114). As a morphological indicator of an ongoing consolidation of epithelial injury, lung tissue of COVID-19 patients could be characterized by an accumulation of fibrin in the alveolar wall and airspaces (74). Of note, pulmonary ILC2s and the ILC2-released cytokine IL-13 have been described as potent mediators of collagen deposition, at least in murine models of lung fibrosis (37). In addition, based on analyses in a mouse model of influenza virus infection, ILC2-derived AREG was postulated to protect and restore the airway epithelium upon viral damage (115). Besides the potential involvement of ILCs in the anti-viral immune response directed against SARS-CoV-2, it should also be taken into account that, at least compared to other immune cell fractions, murine ILCs, and especially ST2-negative ILC2s derived from the small intestine, appeared to show a relatively high expression of the SARS-CoV-2 entry receptor ACE2 at the RNA level (116). It will thus be interesting to clarify in future studies whether ILCs might represent a cellular target for SARS-CoV-2 infection and potentially even virus replication. Since an enormous amount of scientific effort is being exerted worldwide to further decipher the pathology of COVID-19, we can expect to achieve improved and more concrete insights into the functional role and potential therapeutic targeting of local ILC pools during the clinical manifestation and/or exacerbation of this threatening and fast-spreading disease very soon.

Although ILCs have been extensively studied in the lung and gut over the last decade, little is known about their role at the mucosal surface of the urogenital tract. So far, helper ILCs have been analyzed in the uterus and decidua only during pregnancy, and here they were suggested to be important effectors initiating tissue remodeling during implantation (48). In particular, ILC3s and ILC1s were described to be involved in the maintenance of early pregnancy (117, 118). Increased frequencies of ILC3s and ILC2s, however, were associated with overwhelming inflammation in preterm labor (119). In order to further validate the functional relevance of ILCs in this context, it will be of crucial importance to gain more detailed insights into the potential underlying ILC-driven effector mechanisms and molecular mediators.

Collectively, extensive work on the role of helper ILCs at mucosal barrier sites in humans has revealed clear associations of defined ILC subsets with various inflammatory and fibrotic diseases. However, some important questions remain not fully answered: are altered ILC frequencies are a cause or consequence of the associated tissue pathology and which molecular mechanisms underlie their numerical and functional regulation?

Local Modulators of ILC Activity

Local Regulation of Human Helper ILC1s

On the one hand, helper ILC1s have been suggested to be important effector cells that fight intracellular pathogens and bacteria in order to maintain tissue homeostasis. On the other hand, altered ILC1 frequencies in CD (15, 53) and COPD patients (55) indicate the involvement of this ILC subtype in chronic inflammation. Careful regulation of ILC1 activity is thus strictly required to allow the secretion of protective effector cytokines but, at the same time, prevent sustained and overwhelming immune activation resulting in pathologic tissue remodeling and chronic injury.

Cytokines represent one of the main regulatory stimuli of innate immune responses (120). In the case of human ILC1s, the pro-inflammatory cytokine IL-12, which has already been well-known for its ability to promote type-1 immune responses (121), turned out to be also of immense importance for the activation of ILC1s and subsequent IFN-γ release (15, 78) as shown in primary human ILC1s purified from tonsils and peripheral blood (15, 78). In accordance with this, ILC1s expressed higher mRNA levels of the IL-12 receptor subunit B2 (IL12-RB2) than ILC2s and ILC3s (15, 55). Especially in combination with IL-2 and/or IL-18, IL-12 was identified as a potent inducer of IFN-γ production in *in vitro* cultured human ILC1s (15, 55). This IL-12 responsiveness was also true for the unique subset of intraepithelial human $NKp44^+CD103^+$ ILC1s, which have been suggested to mirror key cytotoxic features of tissue-resident $CD8^+$ memory (Trm) cells (44, 122). While IL-18 failed to synergize with IL-12 in the induction of $NKp44^+CD103^+$ ILC1-derived IFN-γ production, IL-15 alone and in combination with IL-12 served as an effective stimulus (44). As the main cellular source of IL-12, antigen-presenting cells (APCs) release high amounts of this type-1 cytokine after exposure to bacteria (123). This is of particular relevance in the context of IBD, where intestinal barrier defects lead to increased mucosal infiltration of luminal bacteria (124). In response to the enhanced release of IL-12p70, IL-18, and IL-1β by local myeloid dendritic cells (DCs), intestinal ILC1s are able to secrete increased levels of the pro-inflammatory cytokines IFN-γ and TNF-α and thus relevantly support the mucosal immune response against bacterial intruders. This was true for gram-negative commensals and pathogens, e.g., *Acinetobacter junii* and *Salmonella typhimurium*, as shown in *in vitro* co-cultures of human ILC1s and lamina propria mononuclear cells (LPMCs) (79). Without proper regulation of this response, chronic inflammation can be established. In CD patients, for example, LPMCs showed hyperresponsiveness toward bacterial components, resulting in enhanced IL-12 levels (125, 126), which was associated with increased accumulation of IFN-γ-expressing ILC1s in the inflamed mucosa (15, 53). Besides monocytes and DCs, co-culture experiments demonstrated that epithelial cells were also able to translate luminal danger signals, such as TLR2, into a stimulatory trigger for human intraepithelial $NKp44^+CD103^+$ ILC1s to produce IFN-γ. Thus, efficient pathogen-mediated activation of intraepithelial ILC1s might even occur in the absence of epithelial barrier destruction (44). To control this, TGF-β was identified as a negative regulator of ILC1-mediated IFN-γ, but not TNF-α secretion (80), a mechanism dysregulated in IBD patients (127).

In addition to the here-described stimuli (summarized in **Table 1**), human blood or tissue ILC1s have been shown to express further surface receptors, such as IL-4R, IL-9R, and ICOS (55), that potentially transmit regulatory signals. This, however, still has to be validated functionally for human ILC1s, and further research will thus be necessary to fully decipher the mechanisms regulating ILC1 activity in humans.

Local Regulation of Human Helper ILC2s

ILC2s and their role in physiological and pathological processes have been extensively studied (128–131). Based on analyses in the murine system, local ILC2s represent an unusually long-lived cell type (132), which therefore requires tightly controlled effector functions. A complex network regulating the activity of tissue-resident ILC2s has been identified (summarized in **Table 1**). This includes soluble mediators, such as cytokines and lipid mediators, as well as direct cell-cell interactions.

Soluble modulators of human ILC2s

Among numerous cytokines, the alarmins IL-25, IL-33, and thymic stromal lymphopoietin (TLSP) constitute the central activation unit of ILC2s (18, 55, 60, 68, 81). It is noteworthy that cytokine-mediated ILC2 activation was accompanied by elevated receptor expression of ST2, IL17BR, and TSLPR on the cell surface, enabling alarmins to further potentiate their stimulatory effects (81). Successful *in vitro* stimulation of human ILC2s was reflected in characteristic morphological alterations, an activated phenotype (68), and increased survival and proliferation of stimulated ILC2s (81). Probably most relevant, stimulated ILC2s showed enhanced effector functions in the form of the secretion of large amounts of type-2 effector cytokines, including, primarily, IL-13 and IL-5, but also IL-4 and GM-CSF (18, 55, 60, 68, 81). In multiple *in vitro* stimulation experiments with primary human ILC2s and stable ILC2 cell lines, combinations of multiple cytokines turned out to induce ILC2 activation most potently. Interestingly, TSLP alone or in combination with IL-25 and IL-33 harbored the highest pro-survival capacity (81), whereas IL-33 appeared to be an important co-factor for the induction of ILC2 proliferation in different cytokine combinations (81). Regarding the effector functions, several studies reported that IL-25, IL-33, and TSLP alone had no or only suboptimal effects on the secretion of selected effector cytokines (18, 60, 82, 92) but displayed synergistic effects with IL-2 (18, 81) or in combination with each other (54, 68, 81). IL-2 is well-known for its pro-survival effects on lymphoid cells; however, it represents a sufficient ILC2 stimulus only in the presence of synergistic co-factors (18, 55, 68, 81, 82). Several studies indicated that the combined effects of IL-25, IL-33, TSLP, and IL-2 represented the most potent stimuli for ILC2 activation (18, 60, 68, 81) and might also resemble the *in vivo* situation very closely. In line with the role of ILC2s as early mediators of mucosal defense, epithelial cells responding to stress signals represent very prominent local sources of the alarmins IL-25, IL-33, and TSLP (133). For example, in patients suffering from chronic rhinosinusitis, nasal

polyp epithelial cells expressed TSLP, which directly activated local ILC2s (18). Besides the epithelium, relevant expression of alarmins could also be detected in endothelial cells, Th2 cells, mast cells, fibroblasts, and macrophages (134–139). So far, most human studies have been conducted with peripheral blood ILC2s. However, tissue-resident ILC2s derived from nasal polyps (18), fetal gut (60), and tonsils (82) showed a similar activation behavior. Further evidence comes from murine experiments showing the ability of IL-33 and IL-25 to boost ILC2 responses *in vivo* (131).

While most of the common ILC2 activators, like IL-33, IL-25, and TSLP, belong to the group of epithelial-derived alarmins and are released upon various stress conditions, IL-1β represents an inflammasome-dependent pro-inflammatory cytokine well-known to trigger fever and the mobilization of neutrophils (140, 141). Although an ILC-activating effect of IL-1β has first been described for the ILC3 compartment (142), marked expression of the IL-1β receptor and respective IL-1β responsiveness were also observed in human ILC2s (55, 60). At least in experiments performed *ex vivo*, IL-1β and IL-1α together with IL-2 served as potent stimuli for human blood ILC2 proliferation and the production of IL-5 and IL-13 (55, 81, 82). In addition, IL-1β was shown to increase the expression of ST2L, IL-17RB, and, to a lesser extent, TSLPR on human ILC2s (82), which was suggested to be a priming signal enhancing the effect of epithelial cell-derived alarmins and explaining their additive effect (82). However, as further discussed later in this article (see paragraph on ILC Plasticity and Tissue Differentiation), other studies indicated that IL-1β might support ILC2-to-ILC1 plasticity (82). Within tissues, activated IL-1β is mainly released by macrophages, DCs, and neutrophils, classically after exposure to Toll-like receptor ligands or DAMP (141), and increased levels of this cytokine could be observed in the lungs of COPD patients, in the inflamed intestinal mucosa of IBD patients and in lesions of autoimmune and inflammatory skin diseases (143–145).

In vitro stimulation with the pro-inflammatory cytokine IL-18 could additionally induce cytokine secretion in human blood ILC2s via the IL-18R (146). Interestingly, in mice, a skin ILC2 subset was identified to preferentially respond to IL-18. These data clearly pointed to the existence of tissue-specific ILC subsets with unique receptor profiles and thus distinct abilities to respond to environmental stimuli (147). In line with this, IL-18 is thought to be involved in various inflammatory cutaneous diseases, including atopic dermatitis (148), suggesting a potential role for active ILC2s in these diseases as well.

The TNF superfamily member TL1A has been suggested as another potent activator of human ILC2s that express high levels of its receptor DR3 (death receptor 3; TNFRSF25) (28). Primarily secreted by alarmed epithelial, endothelial, and myeloid cells, TL1A induced effector cytokine secretion by human ILC2s and acted additively to IL-25 or IL-33 *in vitro*. Murine *in vivo* experiments further revealed the functional importance of this TL1A-driven ILC2 activation in regulating helminth infections and driving type-2 lung inflammation (28).

In combination with external stimulation, IL-4 (55) and IL-9 (29, 149) have been suggested to further boost proliferation and cytokine secretion of activated ILC2s in an autocrine fashion. In the case of IL-4, this autocrine loop could be functionally proven in *ex vivo* stimulated human blood ILC2s (55). Regarding IL-9, the direct functional proof is still restricted to murine data showing the importance of IL-9-driven ILC2 stimulation for the maintenance of lung homeostasis (29, 150) as well as for the resolution of arthritis (149). In accordance with the latter, association data from patients with rheumatoid arthritis showed an inverse correlation between blood ILC2 counts and disease activity (149). Moreover, the chemokine CCL1 could recently be identified as another autocrine activator of ILC2 function in mice and men, mediating its effects via CCR8 signaling (86).

In order to dampen overwhelming ILC2 activity, negative regulators are inevitable to guarantee controlled immune responses. However, our understanding of those immunological mechanisms limiting ILC2-mediated pro-inflammatory effects still remains imprecise, particularly in the human system. Most extensively studied so far, the anti-inflammatory cytokine IL-10 was identified to also suppress the type-2 immune response induced by *ex vivo* stimulated ILC2s (80, 83). IL-10 is secreted by various immune cell types (e.g., macrophages, myeloid DCs, and specific Th cell subsets) (151) and can also be produced by all ILC subsets (80), suggesting mutual control. The potent induction of IL-10 was also described as an important effector mechanism underlying the immunomodulatory properties of IL-27 (152). However, murine studies revealed an additional direct inhibitory effect of IL-27 on ILC2s (50, 131), although the translation of these findings into the human system is still lacking. As another potentially regulatory cytokine, the suppressive function of TGF-β on the cytokine secretion of human ILC2s has been discussed, though controversially (80, 83), with the implication that its described inhibitory effects on ILC2s are dependent on experimental conditions, such as cytokine concentrations and stimulation protocols. Despite IL-10, IL-27 and potentially also TGF-β, type-I interferons and IFN-γ were able to efficiently regulate ILC2 activity in the murine ILC2s *in vitro* and *in vivo* (50, 84, 85, 131, 153). Although the translation of these data into the human system still remains incomplete, the impact of the type-I interferons IFN-α and IFN-β on the activation of regulatory pathways and the downregulation of type-2 cytokine production could successfully be confirmed for human ILC2s, respectively (84, 85).

Next to the active contribution of cytokines, lipid mediators represent another group of immuno-modulatory substances that regulate ILC2 activity, including the arachidonic acid metabolites prostaglandins, leukotriens, and lipoxins.

Most prominently, prostaglandin D_2 (PGD_2) has been shown to activate ILC2s via its G protein-coupled receptor CRTH2 (87, 88), which represents a classical marker for identifying human ILC2s (60). Large amounts of PGD_2 are typically released from IgE cross-linked mast cells during an allergic reaction, resulting in the secretion of pro-inflammatory cytokines by ILC2s as well as the induction of IL-33R expression, further boosting the inflammatory response (87). A more recent study even described an auto- or paracrine stimulatory effect of ILC2-derived PGD_2 (154). Given the increased pulmonary PGD_2 levels

observed in asthmatic and chronic rhinitis patients (155, 156), this might further explain the active contribution of ILC2s in allergic diseases.

In contrast to the activating properties of PGD_2, PGI_2 was assumed to restrict ILC2 effector functions. This was based on the *in vitro* finding that the PGI_2 analog cicaprost reduced IL-2- and IL-33-induced type-2 cytokine production in human blood ILCs. The *in vivo* relevance of this finding was demonstrated in mice with allergen-induced lung inflammation, which displayed reduced pulmonary ILC2 counts after cicaprost treatment and a dependency on PGI_2 receptor signaling (90). However, further proof is necessary to validate these initial findings. Furthermore, another study indicated an inhibitory effect of PGE_2 on human blood and tonsilar ILC2s mediated via the E-type prostanoid receptors (EP) 2 and EP4. In the presence of PGE_2, alarmin-induced secretion of IL-5 and IL-13, expression of GATA3 and CD25, and ILC2 proliferation turned out to be significantly decreased. EP2 and EP4 receptors might thus represent promising target structures for a potential therapeutic modulation of the overwhelmingly activated ILC2 axis in allergic diseases (91).

Following the detection of functional cysteinyl leukotriene receptor 1 (CysLTR1) expression on human blood ILC2s, the receptor ligands LTC_4, LTD_4, and LTE_4 have been identified as additional activators of human ILC2s. In particular, LTE_4 was described as a potent stimulator of ILC2 viability and effector cytokine secretion, with IgE cross-linked mast cells being one of its main producers *in vivo*. Regarding the complex multifactorial situation of tissue inflammation, the alarmins IL-25, IL-33, and TSLP and also PGD_2 were found to amplify the LTE_4-induced effector-cytokine secretion. The CysLTR1 antagonist montelukast, which is clinically approved, for instance, for asthma therapy, was able to inhibit this LTE_4-induced ILC2 activation (89). Interestingly, PGD_2 and the cysteinyl leukotriens LTC_4, LTD_4, and LTE_4 not only activate ILC2s but also harbor chemotactic potential, driving the accumulation and thereby the numerical regulation of local ILC2s (for more details see the chapter Tissue-Specific Migration of ILCs During Adulthood) (87, 89).

Another class of lipid mediators, the lipoxins, are known for their pro-resolving function (157). In line with this, lipoxin A4 has been described to suppress cytokine-induced IL-13 release from human blood ILC2s via the ALX/FPR2 receptor (88).

In addition, the active metabolites of vitamin A and D were found to significantly influence the effector cytokine secretion of human blood ILC2s. While the vitamin A metabolite retinoic acid enhanced the secretion of IL-5 and IL-13 by activated ILC2s as well as the expression of α4β7, the vitamin D metabolite 1,25D exhibited suppressive functions (92).

In the last decade, intense research on human ILC2s has discovered a broad regulatory network mainly consisting of cytokines and lipid mediators controlling human ILC2 activity. If dysregulated, reduced or overwhelming ILC2 responses might lead to parasitic infections and chronic inflammation, respectively (129, 158). Serving as central activators or suppressors of ILC2 responses, the identified soluble mediators and their respective receptors might be of high therapeutic relevance in ILC2-driven diseases. Hence, the identification of further mechanisms regulating ILC2 activity in humans is of great clinical value. Results from murine studies suggest additional classes of potent ILC2 mediators, including hormones and neuropeptides, as well as exogenous agents, like bacterial products, that might serve as potential therapeutic targets. Dihydrotestosterone, a metabolite of the sex hormone testosterone, for instance, was suggested to restrict ILC2 differentiation via androgen receptor signaling, resulting in reduced lung ILC2 numbers in male compared to female mice, both in steady-state and upon allergen-induced lung inflammation (159, 160). This might potentially explain the increased prevalence of asthma in adult women compared to men (160). Moreover, with the identification of the inhibitory impact of β2-adrenergic receptor signaling on murine ILC2 proliferation and activity (161), a new interesting field of ILC2-neuronal cross-talk has been opened up. This was further expanded by the description of the neuropeptides neuromedin U and calcitonin gene-related peptide (CGRP) as efficient positive and negative regulators of murine ILC2s, respectively (162–165). Furthermore, exogenous mediators, including, for example, bacterial products upon infection (153), have been suggested to alter murine ILC2 activity. To serve as potential therapeutic targets, however, translation of these results into the human system and deeper research on the behavior of human ILC2s is mandatory.

Collectively, a plethora of soluble ILC2 regulators have already been identified. Their importance for ILC2 activation or inhibition, however, might vary depending on the tissue-specific phenotype and function of ILC2s (146, 147). A more detailed analysis of organ-specific ILC2 regulation will therefore help to evaluate the potential of ILC2 regulators as therapeutics targets in future.

ILC2-cell interactions

Whereas numerous soluble mediators have been identified that modulate the activity of human ILC2s, they can also be regulated by direct cell-cell interactions with other immune cells, endothelial cells, and stromal cells, in total providing a tight control network (**Figure 1**).

Originally known to mediate firm contact between circulating immune cells and the vascular endothelium and thereby initiating the homing process of lymphocytes into tissues, intercellular adhesion molecules, like ICAM-1 (intercellular adhesion molecule 1) and its integrin ligand LFA-1 (leukocyte function-associated molecule-1), can also provide stimulatory signals between immune cells. Interestingly, human blood ILC2s turned out to express both ICAM-1 and LFA-1, suggesting a potential interaction of ILC2s with each other. And indeed, ICAM-1–LFA-1-mediated contact of human ILC2s efficiently induced IL-5 and IL-13 secretion *in vitro*, which could be significantly diminished in the presence of ICAM-1 or LFA-1 blocking antibodies (93). Upon stimulation by IL-33, which is rapidly released by epithelial cells sensing stress signals *in vivo* (166), ICAM-1 expression was upregulated in human ILC2s (93), indicating the importance of this interaction for mounting efficient ILC2 responses. Using a mouse model of IL-33-induced lung inflammation, the pathophysiological *in vivo*

relevance of this interaction could be strengthened: blocking the CD11a subunit of the ligand LFA-1 resulted in decreased signs of lung inflammation in immunodeficient Rag1$^{-/-}$ mice (93). Experiments with murine ILC2s further elucidated GATA3 and subsequent ERK signaling as a central downstream mechanism of ICAM-1–LFA-1-mediated ILC2 activation (93). ICAM-1-expressing endothelial cells represent further potential interaction partners of LFA-1^{+} ILC2s, and data from the murine system suggested that the LFA-1 subunit β2 drove the migration of blood ILC2s into the inflamed lung tissue (167). Having shown that human ILC2s express functional LFA-1 (93), this might also be relevant in the human ILC2 lung homing process.

Furthermore, co-stimulatory signals have been described to markedly contribute to ILC2 activation, including molecules of the TNF receptor as well as the B7-CD28 superfamilies. For the TNF receptor superfamily member GITR (glucocorticoid-induced TNFR-related protein) and its ligand GITR-L, for instance, a substantial role in ILC2 activation has been indicated (94). Whereas, GITR-L is primarily expressed by APCs and endothelial cells (168), murine and human ILC2s expressed functional GITR that, upon binding to GITR-L or respective agonists, induced ILC2 proliferation as well as upregulation of effector cytokine transcripts (94). Based on murine data, the stimulatory effect of GITR engagement was based on its synergistic effect with IL-33 on the induction of IL-9 expression, which, in turn, upregulated IL-5 and IL-13 in an autocrine, STAT5-dependent fashion (94). In line with this, the interaction of GITR and its ligand appeared to be important for the pulmonary development of allergic inflammation (94). Moreover, human blood and nasal polyp ILC2s were found to express RANK (receptor activator of nuclear factor κ B), another member of the TNF receptor superfamily, which was suggested to be of biological importance in chronic rhinosinusitis patients with nasal polyps. In this context, the ligand RANK-L was mainly expressed by CD45^{+} immune cells, including Th2 cells, and its levels were significantly increased in nasal polyps. Successful RANK–RANK-L engagement stimulated human ILC2s to secrete enhanced IL-5 and IL-13 levels via NFκB signaling and acted in synergy with TSLP (95). Given the stimulatory effects of TNF receptor superfamily members expressed by ILC2s on the induction of type-2 airway inflammation, they might present promising new therapeutic targets in the future. The B7-CD28 superfamily member ICOS and its ligand ICOS-L, which have been described extensively as co-stimulatory molecules in the antigen-specific interaction between Th cells and APCs (169, 170), have also been described as potent auto-stimulatory triggers for the antigen-independent activation of ILC2s. Both functional ICOS and ICOS-L are expressed by human blood ILC2s, where their cell contact-dependent interaction induced a significantly increased production of IL-5 and IL-13 by *in vitro* stimulated ILC2s. In line with this, experimental *in vitro* and *in vivo* blockade of ICOS signaling markedly inhibited the pro-inflammatory properties of human ILC2s, resulting in reduced airway inflammation in a humanized mouse model of IL-33-induced airway hyperreactivity (96). Accordingly, increased numbers of ICOS^{+} ILC2s were detected in the BAL of patients with idiopathic pulmonary fibrosis compared to control subjects (37), further indicating a relevant function of ICOS signaling in ILC2s in the diseased lung. Surprisingly, another study demonstrated that the inhibitory effect of induced Tregs on human ILC2 activity could be blocked efficiently by ICOS-L neutralizing antibodies *in vitro* and *in vivo*. The authors thus postulated a direct interaction between ICOS-L^{+} ILC2s and ICOS^{+} induced Tregs that efficiently suppressed ILC2 effector functions and might therefore act as crucial mediators for the resolution of lung inflammation (97). Taken together, ICOS-L signaling might have contrary roles in human ILC2s depending on the ICOS-expressing interaction partner, which might potentially compete for contact with ICOS-L^{+} ILC2s. (171). Moreover, data from murine experiments suggest that ICOS-L-expressing DCs might serve as an additional interaction partner for ICOS^{+} ILC2s and thus support allergic lung inflammation (172).

Besides their essential molecular involvement in the process of antigen presentation by professional APCs, MHCII molecules have also been shown to be expressed by non-classical APCs, including human ILC2s (173). Oliphant and colleagues detected the expression of both the MHCII molecule HLA-DR and the co-stimulatory CD28 ligands CD80 and CD86 on the surface of human blood ILC2s, allowing the efficient processing and presentation of antigens to Th cells *in vitro* (34). The functional relevance of this observation was further analyzed in the murine system, showing a reciprocal, MHCII- and CD80/CD86-dependent crosstalk between antigen-presenting ILC2s and Th cells in the presence of the cognate antigen that was important for the successful expulsion of *Nippostrongulus brasiliensis* infections. This interaction not only led to the activation of antigen-specific T cells but also triggered ILC2 expansion and IL-13 production via T cell-derived IL-2. ILC2 stimulation was therefore suggested to be initiated by the epithelial cell-derived alarmins IL-25 and IL-33 but to be maintained by IL-2 secreted by T cells upon MHCII–TCR interaction with ILC2s (34).

Originally, the natural cytotoxicity receptor NKp30 was identified as an activating receptor on NK cells mediating the elimination of tumor and virus-infected cells (174). However, it was also found to be highly expressed on blood and *ex vivo* cultured human ILC2s. Upon interaction with the plate- or membrane-bound NKp30 ligand B7-H6, NKp30^{+} ILC2s were stimulated to secrete increasing amounts of IL-13, while the mRNA expression of important activating receptors, including ST2, CRTH2, and IL-17RB, was downregulated. This was suggested to serve as a negative feedback mechanism regulating the activation status of pro-inflammatory ILC2s. Besides tumor cells, B7-H6 could also be detected on the basal epidermis of healthy individuals and even in the suprabasal epidermis layers of atopic dermatitis patients, implying a role of NKp30–B7-H6 signaling in the activation of human skin ILC2s during chronic inflammation (98). B7-H6 expression was additionally found in some tumor samples as well as adjacent normal lung tissue (175), suggesting a potential role in the activation of pulmonary ILC2s, as well. Whether the NKp30-mediated ILC2 stimulation can also be induced by other NKp30 ligands like BAT3 or BAG6 still needs to be clarified.

While a variety of cell-cell contact-dependent ILC2 activators have been identified, there is considerably less understanding of interactions limiting ILC2-mediated inflammation.

Being known as a cell adhesion molecule that provides intercellular junctions between epithelial cells and thereby guarantees a stable barrier as the first line of physical immune defense, E cadherin (epithelial cadherin) can also interact with KLRG1 (killer cell lectin-like receptor G1)-expressing immune cells (176, 177). Interestingly, upregulation of KLRG1 surface expression was observed in human skin ILC2s under inflammatory *in vitro* and *in vivo* conditions. On a functional level, binding of KLRG1 to its ligand E cadherin resulted in significantly decreased proliferation and effector cytokine expression of human ILC2s *in vitro* (54). In the pathological context of atopic dermatitis and asthma, this suppressive mechanism was suggested to be impaired based on reduced local expression of E cadherin in both diseases (54, 178), which finally results in ILC2-driven chronic inflammation. Due to the rather broad expression profile of the adhesion protein E cadherin in different epithelial organs (179–181), the suggested KLRG1-dependent mechanisms might represent an important activation-induced negative feedback loop allowing the termination of local ILC2 responses at different sites of the human body.

The controlled resolution of ILC2-driven immune reactions might further be supported by the checkpoint inhibitor PD-1 (programmed cell death protein 1), which was co-expressed by a relevant subset of KLRG1$^+$ human blood ILC2s. Experimental blockade of PD-1 signaling in human ILC2s significantly enhanced their proliferation and IL-33-induced cytokine production via the STAT5 pathway. Together with the *in vivo* finding that functional PD-1 signaling hindered murine ILC2s to efficiently clear *Nippostrongylus brasiliensis* infections, these results implied a role for PD-1 as an important checkpoint inhibitor regulating activated ILC2s. Although the interaction partner of PD-1$^+$ ILC2s has not yet been analyzed in humans, the PD-1 ligands PD-L1 and PD-L2 are classically induced in various immune cell types (182). Murine data even demonstrated that ILC2s themselves can express PD-L1, which was upregulated upon type-2 inflammation. Unexpectedly, PD-L1$^+$ ILC2s stimulated PD-1-expressing CD4$^+$ T cells rather than suppressing them (33), demanding deeper research into the PD-1 and PD-L1 functions in human ILC2s.

Collectively, our current knowledge on the regulation of human ILC2s indicates the existence of a tight network involving numerous control mechanisms but also offering many potential cellular and molecular targets for dysregulation.

Local Regulation of Human Helper ILC3s

By secreting IL-22 and IL-17, helper ILC3s are crucial for preserving the barrier integrity of mucous epithelia and thereby protecting the host against invading pathogens. However, when dysregulated, the host-protective functions of ILC3s can transform into detrimental immune activation, finally leading to chronic inflammation (183–185). So far, ILC3 research has primarily focused on their function in the intestine, where IL-22-expressing ILC3s are present even under steady-state conditions, while only very low numbers of IL-17-producing ILC3s could be detected in the non-inflamed human gut (6).

Classically known from the maintenance of Th17 cells in the adaptive immune system (186), the cytokines IL-23 and IL-1β also represent prototypical inducers of IL-22 secretion by human ILC3s (30, 52, 142, 187) and are mainly released by DCs and epithelial cells upon tissue inflammation (6). Thus, IL-23 and IL-1β serve as potent mediators that translate the intestinal penetration of commensal and pathogenic bacteria into the induction of a tissue-protective immune response initiated by ILC3-derived IL-22 via CD11c$^+$ myeloid DCs. Indeed, human intestinal ILC3s that had been in contact with fecal bacteria in the intestine were characterized by increased IL-22 production *ex vivo* compared to those derived from tissue sites without fecal bacteria exposure (30). Interestingly, this indirect stimulation of human ILC3s by bacteria was more pronounced in intestinal ILC3s than in tonsillar ILC3s (188), suggesting a tissue-specific regulation of this phenomenon. Independent of accessory cell mediators, bacterial products can also directly induce the proliferation and cytokine production of ILC3s via the activation of Toll-like receptor (TLR) signaling, as shown for NFκB-dependent TLR2 activation in tonsillar human LTi ILC3s in the presence of IL-2 (103). Moreover, products of the bacterial tryptophan metabolism are suggested to directly stimulate ILC3s via binding to the aryl hydrocarbon receptor (AHR), which was shown to be expressed on human ILC3s (104, 105). The functional relevance of AHR signaling on ILC3s was later demonstrated in mice in the context of resolution of *Citrobacter rodentium* infection (189). In contrast, other bacterial products inhibit ILC3 activity, likely to delay epithelial repair and favor their own dissemination. A candidate for this is the *Bacillus anthracis* toxin, which could be shown to suppress IL-22 production by IL-23-stimulated human ILCs *in vitro* via MAPK signaling disruption (106).

Under *in vitro* conditions, ILC3 proliferation and IL-22 secretion could also be induced by the combined effect of the survival factor IL-15 and the pro-inflammatory cytokine IL-18. The stimulatory effect of IL-18 was mediated via ligation of the IL-18Rα and IL-18Rβ subunits on the surface of tonsillar human ILC3s, resulting in functional signaling of the heterodimeric IL-18 receptor, subsequent NFκB activation and finally the transcription of the *IL22* gene (100). *In vivo*, IL-18 secretion could be detected in CD11c$^+$ DCs located in direct proximity to ILC3s in human tonsils (100), enabling paracrine ILC3 stimulation. Moreover, increased IL-18 levels (190) together with the enhanced IL-22 secretion observed in ileal ILCs from CD patients (30) indicate a significant role of IL-18 stimulated ILC3s in the pathological context of CD.

Data acquired in a murine model of spontaneous colitis and *in vitro* analyses of human LPMCs additionally demonstrated a certain stimulatory function of the pro-inflammatory cytokine IL-6 on the ILC3-mediated cytokine secretion in the gut (101). Colon explant cultures of IBD and control subjects further detected a subgroup of IBD patients with high IL-6 production compared to controls (101), implying that IL-6 might be, at least partly, responsible for the increase in IL-17-expressing mucosal ILCs observed in a subgroup of CD patients (52).

In addition to its functional impact on ILC2s (28), the DR3 ligand TL1A also acts as a co-stimulatory trigger for the IL-1β- and IL-23-induced cytokine production and proliferation of human ILC3s, as shown in both stimulated intestinal and *in vitro* differentiated human ILC3 cultures (30, 102, 191). Mechanistically, TL1A was suggested to induce the expression of the IL-2 receptor subunit CD25 on TL1A-stimulated ILC3s and thus to prime ILC3s for acquiring proliferative signals via IL-2 (192). The idea of a functional DR3-TL1A interaction on human ILC3s was further strengthened by the finding that human ILC3s expressed DR3 transcripts even under resting conditions (192). Under pathophysiological conditions, microbial-sensing mononuclear phagocytes appeared to be an important source of TL1A. They were thus able to initiate an anti-microbial, tissue-restoring immune response (30) and should be taken into account, especially in the context of IBD. In line with this, inflammatory intestinal tissue sites of IBD patients were characterized by increased levels of IL-22, likely derived from ILC3s (30). In parallel, intestinal inflammation in IBD patients is associated with an accumulation of IL-17$^+$ ILC3s in the ileum and colon and an increased capacity of IL-23 to trigger the expression of IL-17A in gut-derived ILC3s (6, 52).

Besides the here described involvement of cytokines (**Table 1**), mucosal immune cells, and bacteria in the ILC3-activating machinery, several other factors have been suggested to be crucial promotors of local ILC3 accumulation and function in humans, such as neurotransmitters, vitamin metabolites, and even lifestyle (e.g., obesity and cigarette smoking) (107, 108, 193, 194). In the case of neurotransmitters, vagus-derived acetylcholin was described to stimulate the PCTR1 pathway in both murine and human ILC3s, favoring the resolution of inflammation (107). Moreover, the enteric neuron-derived vasoactive intestinal peptide (VIP) was shown, at least in mice, to modulate ILC3 activity upon food intake, though with controversial effects (195, 196). Ingested as a food component or directly synthesized in sun-exposed skin, the active metabolite of vitamin D, 1,25D, was additionally described to alter the transcriptional profile of human ILC3s, skewing them toward the IL-1β pathway while downregulating IL-23R signaling at the same time. In IBD, where vitamin D deficiency has been reported to be a risk factor, the observed beneficial effects of vitamin D substitution (197) might thus mechanistically include the inhibition of IL-23- and IL-17A-secretion by ILC3s (108). In general, only little is clearly known about the mechanisms negatively regulating human ILC3 proliferation and their activity so far. The first hints of potential ILC3 cytokine inhibitors were acquired from *in vitro* stimulated human tonsillar ILC3s only and demonstrated the suppressive effect of recombinant human IFN-α and IFN-γ on ILC3 numbers (100). Future research should therefore intensify its work on the identification of inhibitors regulating ILC3 activity in order to potentially pave the way for novel therapeutic strategies in inflammatory diseases characterized by an overwhelming ILC3 activity, like IBD.

ILC Plasticity and Tissue Differentiation

Since a markedly altered local ILC composition has been described in inflammatory diseases (75, 183, 198–200), it is important to understand the underlying mechanisms as well as the pathological relevance of this observation. Changes in local cell numbers can be explained by cell death, local proliferation, intercompartmental redistribution, or directed recruitment of existing ILC fractions from distal sites. Moreover, the differentiation of tissue-resident ILC precursors and the transdifferentiation of mature ILCs (**Figure 2**) can also contribute to altered ILC numbers in inflammatory tissue sites (**Figure 1**), allowing adaption to local requirements without recruiting additional cells (201). While initial studies identified three helper ILC subsets, a more complex diversity has now been described (10, 104, 202), including intermediates between distinct mature subgroups and ex-ILCs derived from the transdifferentiation of one ILC subgroup into another (202).

ILC3 ↔ ILC1 Plasticity

ILC plasticity seems to be particularly interesting in pathologies characterized by the increase of one ILC subgroup at the expense of another. This was observed, for instance, in CD patients, who are characterized by having enhanced ILC1 frequencies in inflamed intestinal tissue with a simultaneous decrease in the NKp44$^+$ ILC3 population compared to non-inflamed control tissue (15). Searching for a functional link between these associated phenomena, it was important to learn that the type-1 cytokine IL-12 not only represents a potent ILC1 activator (44) but also serves as a key inducer of ILC3-to-ILC1 transdifferentiation. *In vitro* experiments with human tonsillar and fetal gut ILC3s confirmed the plasticity of mature ILC3s, differentiating into IFN-γ-producing ILC1s upon IL-2 and IL-12 stimulation with or without the addition of IL-1β (15, 201, 203). Moreover, TGF-β was also suggested to induce T-bet expression in stimulated human ILC3s (202). *Ex vivo* analyses of human ileal LPMCs revealed the existence of ILC subgroups in the transition phase that share both ILC3 and ILC1 characteristics, thus hinting at a biological relevance of the ILC3-to-ILC1-shift even in the complex *in vivo* situation (202). This was further proven in a humanized mouse model: after adoptive transfer of *ex vivo* expanded IL-22-secreting human ILC3s, an organ- and time-dependent switch to IFN-γ secretion was observed (202). For transition, the transcription factors Aiolos and Ikaros were suggested to shut down the transcription of ILC3 signature genes, thereby allowing the induction of an ILC1-like phenotype and function (202, 203).

Conversely, the combined effects of IL-2, IL-23, and IL-1β induced the phenotype and function of mature ILC3s in former human ILC1s, including RORC expression and IL-22 secretion, which could be further triggered by the vitamin A metabolite retinoic acid. Indeed, the intravenous transfer of human ILC1s into a humanized mouse model resulted in the appearance of human NKp44$^+$ ILC3s in the gut of recipient animals, further proving the *in vivo* validity of the ILC1-to-ILC3 switch even under non-inflammatory conditions (201). In the pulmonary mucosal tissue, the clinical significance of the ILC3-to-ILC1 shift was described in the tumor context in patients with pulmonary squamous cell carcinoma. Induced by IL-23-secreting tumor cells, human lung ILC1s gave rise to functional ILC3s and thereby supported IL-17-mediated tumor growth. This was, in particular,

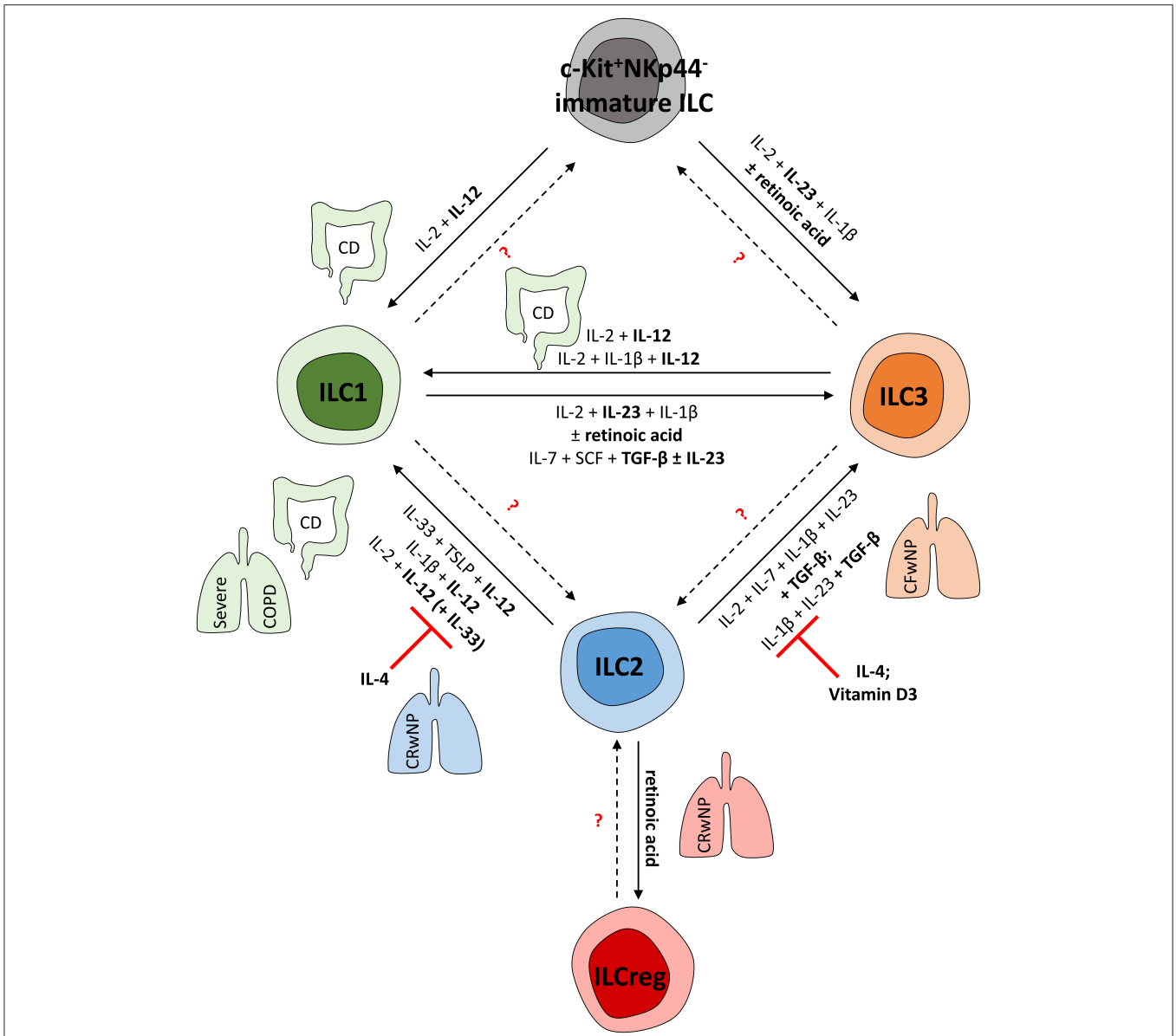

FIGURE 2 | (Trans-) Differentiation of human ILCs in mucosal tissues. To adapt the local pool of tissue-resident ILCs, mature ILCs can differentiate from local precursors or transdifferentiate into other ILC subsets. This shift in local ILC subgroups is mediated by the local cytokine environment and can be observed in various fibro-inflammatory diseases affecting the lung and gut. CD and severe COPD, for instance, are characterized by an ILC1-dominated immune response. In line with this, human ILC1s can be induced from c-Kit^{+}NKp44^{-} ILC precursors as well as from mature ILC2s and ILC3s in these pathologies; this is mainly regulated by IL-12. In contrast, in chronic rhinosinusitis with nasal polyps (CRwNP), ILC2s rather than ILC1s represent important effector cells triggering the immune response. Accordingly, ILC2 plasticity can be suppressed by IL-4 and vitamin D3, hampering the acquisition of ILC1- or ILC3-like characteristics to preserve type-2 immunity. Nevertheless, retinoic acid can trigger ILCreg formation from mature ILC2s, likely representing a negative feedback mechanism to control ILC2 responses. Though characterized by nasal polyps as well, cystic fibrosis patients (CFwNP) display an ILC3-driven phenotype in upper airways that can be induced by TGF-β-driven transdifferentiation of ILC2s into ILC3s. Potentially, ILC1s might also represent a source of mature ILC3s, as shown *in vitro* in response to IL-23, TGF-β, and retinoic acid. Whether further ILC plasticity exists in humans needs to be targeted in future studies.

reflected by the finding that high ILC3 numbers and IL-23 and IL-17 levels turned out to significantly correlate with decreased patient survival (204). An even higher level of flexibility in the ILC1/ILC3 ratio is achieved by the availability of local immature human ILCs (c-Kit^{+}NKp44^{-}), which can undergo differentiation toward either functional ILC1s or NKp44^{+}ILC3s in the presence of IL-2 and IL-12 or IL-2, IL-23, and IL-1β, respectively (15).

ILC2 ↔ ILC1 Plasticity

Another ILC1-dominated disease, COPD, is characterized by enhanced ILC1 frequencies at the cost of ILC2s in the peripheral

blood and also the inflamed lung tissue (55, 200). Since the number of total ILCs was unaffected by the IL-12-enriched inflammation in COPD patients (200), the transdifferentiation of mature ILC2s into functional ILC1s might explain this inverse correlation of ILC1 and ILC2 frequencies, pointing to the ILC1-inducing cytokine IL-12 as a candidate mediator. And indeed, *in vitro* stimulated human ILC2s lost their type-2 characteristics in the presence of IL-12 and acquired the phenotype and function of ILC1s instead (55, 200). Interestingly, IL-1β was found to further support the ILC2-to-ILC1 shift by priming ILC2s for optimal response to IL-12 (82). In particular, a subset of IL-13$^+$ human ILC2s turned out to co-express IFN-γ in response to strong IL-12 signaling (205). Accordingly, ILC2s derived from IL-12Rβ1-deficient patients with mendelian susceptibility to mycobacterial disease were unable to exhibit ILC2-to-ILC1 plasticity while intestinal samples from CD patients harbored transdifferentiated IL-13$^+$IFN-γ$^+$ ex-ILC2s (205). The functional relevance of the described ILC2-to-ILC1 switch for the clinical course of inflammatory pathologies was further confirmed by showing a positive correlation between increased ILC1/ILC2 proportions in COPD patients and augmented symptoms of respiratory disease (200). Chronic exposure to cigarette smoke and respiratory tract infections, known to be strongly associated with the occurrence of COPD, might further trigger this conversion (200). Surprisingly, the classical type-2 cytokine IL-33 was able to enhance the IL-12-induced IFN-γ production in human ex-ILC2s, indicating a dual, context-dependent role of IL-33 (200). In contrast, IL-4 acted as a classical type-2 cytokine and could reverse human ex-ILC2s into functional ILC2s again *in vitro* and might thus be able to support the maintenance of an ILC2 predominance in mucosal tissues, as observed in patients suffering from chronic rhinosinusitis with nasal polyps. An increased proportion of ILC2s was detected in the turbinate tissue of these patients, while the local frequencies of ILC1s and ILC3s were diminished. This observation might be partly explained by the co-localization of ILC2s with IL-4-secreting eosinophils and the capacity of IL-4 to stabilize the phenotype and function of ILC2s (55). However, besides the described capacity of IL-4 to re-convert ex-ILC2s to their initial ILC2 phenotype, there have been no reports describing milieu-dependent transdifferentiation of human ILC2s from bona fide ILC1s or ILC3s (55). Also, in the clinical context of chronic rhinosinusitis, nasal polyps of affected patients showed an increased frequency of ILCregs (206). Similar to Tregs, their counterparts in the adaptive immune system, ILCregs possess a regulatory capacity exerted via the secretion of the immunosuppressive cytokine IL-10 (22). Interestingly, ILC2-to-ILCreg transdifferentiation seemed to appear in the presence of retinoic acid and resulted in marked IL-10 secretion by former human ILC2s (206).

ILC2 ↔ ILC3 Plasticity

Unlike the type-2 signature dominating the immune response in nasal polyps of patients with chronic rhinosinusitis, cystic fibrosis patients with nasal polyps exhibited a substantially increased frequency of NKp44$^-$ ILC3s compared to chronic rhinosinusitis patients, even though nasal polyps in the two diseases share morphological and clinical characteristics (72). Based on *in vitro* data showing the transdifferentiation of human ILC2s into ILC3-like cells in the presence of TGF-β, an ILC2-derived IL-17-secreting ILC3-like subtype was suggested to be responsible for this observation (72, 207). Consistently, increased TGF-β levels have been described in nasal polyps of cystic fibrosis patients (72). In this context, epithelial cell-released TGF-β was suggested to induce SMAD2/3 phosphorylation in nasal human ILC2s and thereby initiate their transdifferentiation into IL-17A-secreting ILC3s. In turn, ILC-derived IL-17A can recruit neutrophils and thus further promote inflammation (72). A similar switch of the c-Kit$^-$ ILC2 subgroup was observed in psoriatic skin lesions, identifying mutual control of GATA3 and RORγt expression as an important control center deciding the fate of ILC2s (207). Through cell culture experiments, IL-4 and vitamin D3 could be revealed as antagonists of this ILC plasticity, suppressing the TGF-β-initiated subtype switch (72, 207). The biological impact of this ILC2-to-ILC3 conversion, however, is restricted to the skin (207) and upper airways (72) so far, and whether this also applies to the lower airways and other organs needs to be addressed in future studies (202). In addition, it is still insufficiently clarified whether ILC2s can fully convert into ILC3s or whether they might keep certain ILC2 characteristics as ILC3-like cells (207). This also raises the question of whether multistep ILC plasticity is possible or whether there are specialized subsets of ILC1s, ILC2s, and ILC3s that can only adapt defined characteristics of another subgroup.

Although experimental proof of ILC3-to-ILC2 plasticity in humans is lacking to date, it was interesting to note the identification of lin$^-$CD117$^+$CD127$^+$ LTi-like cells as an intermediate subset between LTi ILC3s and functional ILC2s. Assuming that there was no contamination of this cell population with mature ILC2s, simultaneous production of the type-3 cytokine IL-22 and the type-2 cytokines IL-5 and IL-13 has been demonstrated in response to PMA, ionomycin, and brefeldin A in expanded human CD127$^+$ LTi-like cells. Moreover, clonal expansion of LTi-like cells revealed heterogeneous effector cytokine profiles of analyzed clones, which were skewed either to the type-3 or the type-1 side but showed comparable RORC and GATA3 levels (103). Thus, LTi-like cells might represent an intermediate or precursor ILC subset. Stimulation with IL-2 or IL-15 and the TLR2 ligand Pam3 increased the IL-13 and IL-22 secretion by human LTi-like cells *in vitro*, while only the minority of cells were IL-22$^+$ (103), indicating that bacterial products might directly activate LTi-like cells and, in combination with further stimuli, might decide the fate of this intermediate ILC subset. Further research, however, is necessary to confirm the direct link between the described LTi-like cells and the transdifferentiation of ILC3s into ILC2s. In another study, KLRG1$^+$ ILCs were additionally suggested as intermediate cells biased toward the ILC2 lineage but with the potential to differentiate into IFN-γ- and IL-22-producing ILCs upon stimulation with IL-1β and IL-23 (208). Similarly, NKp46$^+$ ILCs were postulated to represent ILC3 precursors but with the ability to generate ILC1- and NK cell-like ILCs upon IL-12 treatment (208).

Collectively, human ILCs have been described as highly plastic cells (**Figure 2**). Indeed, many key cytokines regulating

ILC activity have been demonstrated not only to control the proliferation and effector cytokine secretion of a distinct ILC subgroup but also to mediate the transdifferentiation of ILCs. Thus, dependent on the environmental stimuli, the plasticity of mature ILCs and the differentiation of local ILC precursors enable a rapid and reversible adaption of the ILC pool to local requirements and subsequent modulation of the innate immune response.

Tissue-Specific Migration of ILCs During Adulthood

In humans, relatively small but distinct populations of ILC precursors and even mature ILCs are present in the peripheral blood stream during child- and adulthood (26, 51, 80, 208). Yet, their functional role in the circulation itself or on local immune responses has not been fully elucidated. Since disease-associated tissue inflammation, as observed for instance in asthma, is not only reflected in an adapted local ILC pool but also in altered ILC frequencies in the peripheral blood (31, 64), a biological impact of circulating blood ILCs on systemic or local immune responses is strongly suggested. Functional data acquired in parabiotic mouse models initially argued against this, establishing a paradigm of tissue-resident ILCs that are, at least in the murine organism, incapable of homing from the blood stream to the inflamed tissue site (49, 50). Nowadays, the concept of a strict tissue residency of ILCs has become outdated, superseded by the idea of a rather time- and context-dependent homing capacity of blood ILCs (209) as a response to steady-state losses or under inflammatory conditions. Even in the model of parabiotic mice, a small but significant homing of blood ILC2s into tissues could be overserved upon chronic inflammation (49). In line with this, a recent study described an infection- and inflammation-triggered interorgan migration of gut-resident ILC2s via S1P-mediated chemotaxis in mice. In particular, intestinal inflammatory ILC2s were identified to be a migratory ILC subset that played a crucial role in clearing helminth infections and restoring epithelial tissue integrity, not only in the gut but also in the distant lung tissue (35). Regarding the translatability of the concept of trafficking ILCs into the human system, expression of functional S1P receptors was also proven in human tonsillar ILC1s, ILC2s, and ILC3s. *In vitro*-performed chemotaxis assays further confirmed active migration of human ILC1s and ILC3s in response to S1P analogs with a prominent role of S1PR1, while ILC2s were not analyzed in this context. *In vivo* therapy of patients with relapsing-remitting multiple sclerosis with the S1P agonist fingolimod resulted in an impressive reduction of all ILC subgroups in the peripheral blood, suggesting S1P-dependent trafficking of blood ILCs into lymph nodes in the human *in vivo* situation, too (210). In a completely different clinical setting, partial repopulation of ILC niches with ILCs after myeloablation was shown to take place postnatally in patients with severe combined immunodeficiency after hematopoietic stem cell transplantation (26). These results indicated the migration of donor-derived ILCs or their precursors to replenish blood and tissue ILCs even after birth and fit very well with the recently postulated model of tissue-specific "ILC-poiesis." With the identification of uni- and multipotent $CD117^+$ ILC precursors in peripheral human blood, milieu-driven recruitment and local maturation of blood-derived ILC precursors has been suggested to replenish and adapt the pool of tissue-resident mature ILCs (51). As well as these circulating $CD117^+$ ILC precursors, mature ILCs also exist in the blood stream (26, 60, 80) and show a characteristic surface expression profile of chemokine receptors and integrins, which are generally known as key regulators of tissue-specific homing processes (211). Circulating ILC1s expressed varying levels of CCR4 and CCR6 and were mainly characterized by high frequencies of $CCR7^+$, $CXCR3^+$, and $\alpha4\beta7^+$ cells but lower percentages of $CCR5^+$, $CCR9^+$, and $CXCR6^+$ ILC1s (53, 212). In contrast, the vast majority of human blood ILC2s expressed CCR4, CCR6, and the integrins α4, αL, β1, and β2 and additionally displayed distinct but smaller subsets of $CCR9^+$ and β7 $integrin^+$ cells (53, 167, 212). Only rare ILC2 subsets expressed CCR5, CCR7, and CCR10, while CXCR3, CXCR5, and CXCR6 were almost absent on blood ILC2s (53, 212). While expressing CCR4 and CCR6 in varying levels as well (53), human blood ILC3s differed from other helper ILCs by the expression of CCR10 and cutaneous lymphocyte antigen (CLA) (212). However, they also showed small fractions that stained positive for CXCR3, CCR7, and α4β7 integrin (53, 212). In adaptive immune cells, several of those chemokine receptors have been inferred to drive organ-specific homing pathways that might be translatable to ILCs as innate counterparts of Th cells. CCR7, for example, is known to drive homing to lymphoid tissues, while α4β7 integrin and CCR9 are specific for intestinal migration (213). CLA was suggested to promote homing processes to the skin (214). Whether this concept actually applies to human blood ILCs, however, needs further validation on a functional level. As a first step, the general homing capacity of human blood ILCs could be demonstrated in humanized mouse models: intravenously injected human ILCs could later be detected in various organs as tissue-resident cells (55, 201). Moreover, *in vitro* chemotaxis assays further elucidated specific ligand-receptor interactions regulating the controlled attraction of ILCs. Most prominently, the PGD_2 receptor CRTH2 not only serves as phenotypical hallmark and activating receptor on ILC2s but could also promote directed *in vitro* migration of ILC2s toward PGD_2 (87, 215, 216). Activated, IgE cross-linked mast cells were detected to be a major source of PGD_2, suggesting a relevant role of the CRTH2–PGD_2 interaction for mast cell-induced ILC2 recruitment upon allergic inflammation (87). In line with the accumulation of ILC2s in asthmatic lung tissue (31), ILC2s derived from asthmatic patients displayed enhanced migratory potential toward PGD_2 compared to ILC2s from healthy subjects (216). The *in vivo* relevance of this CRTH2-driven ILC2 migration was underlined in mice confirming efficient PGD_2-mediated accumulation of murine ILC2s in the lung and the importance of CRTH2 for efficiently mounting an anti-helminth lung inflammation (217). Likewise, the leukotriens LTE_4, LTD_4, and LTC_4 also displayed chemotactic potential on human blood ILC2s, with LTE_4 being the most potent chemotactic trigger when tested *in vitro* (89). Though less potent than PGD_2 or LTE_4 (87, 89), IL-33 could also trigger *in vitro*

ILC2 migration (54, 87, 215). In contrast, other members of the ILC2 core activating unit, including IL-25 and TSLP, only showed a minimal chemotactic potential or were effective only at high concentrations, respectively (54, 87). Furthermore, TGF-β and the chemokine CCL8 could also attract human ILC2s in transmigration assays (215), which might be of functional relevance, as an accumulation of ILC2s could be detected in TGF-β-enriched asthmatic airways (218), and IL-33-induced lung inflammation in mice was associated with increased levels of peribronchial CCL8 (215). In tuberculosis-associated lung pathology, a reverse correlation of all ILC subsets has been observed with decreased frequencies in the peripheral blood, but an accumulation of these cells in the affected lung tissue, which was suggested to result from CXCL13–CXCR5-driven ILC lung homing. Thereby, migrated ILC3s were proposed to have a beneficial role against *Mycobacterium tuberculosis* in particular (69).

During the controlled process of immune cell homing, chemokine receptor-mediated signaling is of crucial importance for the activation of integrins expressed by rolling blood cells. Activated integrins mediate the actual adhesion of circulating immune cells to the endothelium (211). Phenotypically, a proportion of human blood and tissue ILC2s has been described to express the integrin subunits α4, αL, β1, and β2 (167). So far, the contribution of αLβ2 rather than α4 and β1 to the ILC2 lung homing process, however, has been functionally proven only in the murine system (167). In the context of gut immune homeostasis, intestinal DCs within mesenteric lymph nodes are specialized for metabolizing dietary vitamin A toward all-trans retinoic acid, which is known to induce membrane expression of α4β7 in $CD4^+$ T cells and thereby imprints T cells for gut homing (219, 220). Thus, it was interesting to observe a similar increase in α4β7 expression on the surface of human blood-derived ILCs after *ex vivo* exposure to retinoic acid. In synergy with IL-2, retinoic acid successfully induced upregulation of α4 and β7 expression in ILC1s, ILC2s, and ILC3s and, in addition, also promoted a significant increase of β1 integrin levels in all three ILC subgroups (92). In contrast to the indicated capacity of retinoic acid to facilitate gut homing of ILCs via binding to the typical intestinal adhesion molecules MadCAM-1 and VCAM-1, vitamin D seems to counteract this effect. The retinoic acid-induced increase in surface expression of α4β7 integrins could be significantly inhibited by the vitamin D metabolite 1,25D in a dose-dependent manner (92).

Besides the influence of chemokines and integrins, the migratory behavior of ILCs might also be modulated by extracellular matrix proteins. In particular, type-I collagen was found to trigger changes in the cytoskeleton of human ILC2s, resulting in increased agility *in vitro*. Type-2 meditated inflammatory diseases of the lung might therefore be amplified by locally recruited and retained ILC2s upon pulmonary tissue remodeling (215).

Apart from the controversial discussion about the tissue residency or systemic mobility of human ILCs, they are assumed to be motile within tissues with a tightly controlled intra-organ localization and spatial distribution (215). But since functional data on human ILCs have been acquired in transmigration assays only, information on chemokines mediating inter- and/or intra-organ migration of ILCs is still lacking. Thus, based on the expression pattern of chemotactic mediators and their receptors, for now, it can only be speculated that, for example, CCR6-driven ILC2 migration might be particularly important for attracting ILC2s from the blood circulation to the tissue, since CCR6 expression is downregulated once ILC2s reside in the lung (221). In contrast, surface expression of integrin αE (CD103), and potentially also CXCR6, seems to predispose human $NKp44^+$ ILC1s for intraepithelial accumulation (44). Interestingly, cell culture experiments indicated that the epithelium itself is able to control the maintenance of integrin αE expression on intraepithelial ILC1s via the release of TGF-β (44). Regarding the intra-organ distribution of ILC3s, the transmembrane chemotactic receptor GPR183 and its ligand 7a,25-dihydroxycholesterol were suggested to play a key role in the organization and localization of ILC3s within mesenteric lymph nodes, which might also be relevant in human GPR183-expressing ILC3s (222).

An augmented occurrence of highly organized ectopic lymphoid aggregates in, for instance, gut, lung, or liver tissue represents a frequently described feature of chronic inflammatory diseases like IBD, COPD, or rheumatoid arthritis, respectively (223). As LTi ILC3s crucially contribute to the formation of ectopic lymphoid aggregates via the secretion of lymphotoxin, IL-17A, and IL-22 (223), it was interesting to find a significantly increased number of neuropilin-1 (NRP1)-positive LTi cells in pulmonary tissue of COPD patients (224). Indeed, the adhesion molecule NRP1 turned out to represent a characteristic marker of human LTi ILC3, which impacts their chemotactic behavior functionally. *In vitro* analyses indicated that the chemoattractant vascular endothelial growth factor A (VEGF-A) was able to induce migration of LTi cells via engagement of NRP1 in complex with VEGFR2 (224). Together with a well-described upregulation of VEGF expression under chronic inflammatory conditions (225, 226), these findings strongly imply that the VEGF-A—NRP1-dependent recruitment of LTi ILC3s is able to trigger the formation of ectopic lymphoid aggregates in inflamed tissue sites and thereby influence the quality of the mucosal immune response (224). Besides their impact on the induction of ectopic lymphoid aggregates, ILC3s might further contribute to the recruitment of ILCs to local sites of inflammation via the release of GM-CSF. A study conducted by Pearson et al. (227) identified circulating and colon-infiltrating ILC3s as a relevant source of GM-CSF in humans and described a significant upregulation of $GM\text{-}CSF^+$ ILC3s in the blood of IBD patients. Based on observations in murine colitis, the inflammation-triggered exit of ILCs from colonic cryptopatches into the adjacent tissue is GM-CSF-dependent and can thus be promoted by activated ILC3s (227). However, this functional link between ILC3-derived GM-CSF and innate immune cell mobilization from ectopic lymphoid aggregates still needs to be confirmed for the human system.

Taken together, more intense research is necessary to validate our current understanding of the systemic and local mobility of human ILCs, as it is still mainly based on phenotypical

observations and *in vitro* findings. Most likely, the availability of humanized mouse models will substantially support us in achieving new insights into the chemotactic stimuli attracting blood ILCs under various *in vivo* pathophysiological conditions. Since our current knowledge of chemotactic ILC attraction has been mainly restricted to ILC2s, upcoming analyses should also include research into the migratory capacity of ILC1s and ILC3s.

CLINICAL IMPLICATIONS

Even though ILC activity is controlled by a tight regulatory network within the human body, dysbalanced ILC frequencies and activity have been observed in the context of numerous diseases characterized by chronic inflammation, fibrosis, or malignant transformation of mucosal tissues (15, 60, 146, 228). Due to their remarkably fast and potent capacity to react to stress signals with the release of immune coordinating effector cytokines, ILCs might represent important target structures for innovative biomarker and treatment strategies. Although our knowledge of ILCs has grown exponentially in the last decade, no ILC-specific application has yet entered the clinics. However, the therapeutic efficacy of several T cell-targeting standard therapies might actually derive from their combined suppressive effects on T cells and ILCs. For instance, glucocorticoid therapy was able to normalize enhanced blood ILC2 frequencies in asthmatic patients (229). Since ILC2s have been suggested to be the main producers of the pro-inflammatory cytokines IL-5, IL-9, and IL-13 in asthmatic patients (229), their contribution to pathologies must not be underestimated. Similarly, systemic glucocorticoid treatment reduced nasal ILC2 proportions in patients with eosinophilic nasal polyps (230). In accordance with these *in vivo* observations, *in vitro* studies confirmed a direct inhibitory effect of the glucocorticoids dexamethasone and budesonide on the cytokine production of activated human blood ILC2s (83, 229, 231), which were proven to express the glucocorticoid receptor (229). Interestingly, this dexamethasone responsiveness turned out to be dependent on the stimuli activating ILC2s. While IL-25- and IL-33-driven ILC2 activities could be successfully suppressed by dexamethasone, this was not the case for IL-7- and TSLP-stimulated human blood ILC2s (231). In line with this, BAL ILC2s derived from asthmatic patients that had been exposed to elevated TSLP levels *in vivo* also displayed dexamethasone resistance (231). Given the elevated levels of both IL-33 and TSLP in the BAL of asthmatic patients (232), the therapeutic efficacy of glucocorticoids might largely depend on the inflammatory microenvironment.

Another commonly used drug in the therapy of asthma, the leukotriene receptor 1 antagonist montelukast (233), is known to relevantly impact the fate of ILC2s. Based on its inhibitory effect on the cytokine production of human skin and blood ILC2s *in vitro* (87, 89), it is reasonable to assume that the *in vivo* efficacy of montelukast is also supported by its ILC2-dampening capacity. In cultured ILC2s, montelukast could further be proven to abrogate the chemotactic and anti-apoptotic potential of cysteinyl leukotrienes (89).

More recently, anti-cytokine therapies have been successfully introduced in the treatment of various inflammatory diseases and partly also target important ILC effector cytokines. For instance, patients with severe nasal polyps showed significantly decreased disease severity upon treatment with the anti-IL-5 antibody mepolizumab (234). Moreover, beneficial effects of anti-IL-4 and anti-IL-13 antibodies have been suggested for a subgroup of asthmatic patients (235, 236). These data strongly imply that treatments originally designed to target T cells and their effector cytokines might additionally function by modifying ILCs. Whether ILCs can also be targeted specifically is unclear to date and requires further research. Based on our current knowledge, however, the partial functional redundancy between ILCs and Th cells under physiological conditions (26) and the crucial impact of mucosal ILCs on multiple inflammatory disorders (6, 75) qualifies this innate cell type as an excellent therapeutic target with minimal adverse events (5, 237).

CONCLUSION

While the explicit benefit of ILCs for healthy individuals has been questioned under the very high hygiene standards in industrialized countries (26), ILCs have been impressively proven to play essential roles in multiple pathologies. In particular, their prime function as guardians and first line of defense at mucosal barrier surfaces makes them a key factor deciding between the induction of controlled and protective or overwhelming and detrimental immune responses upon pathogen entry. Thus, a tight regulation of ILC numbers and their activity is highly important. Indeed, a dense network has been identified that regulates human ILCs, consisting of soluble factors as well as cell contact-dependent processes. These mediators can directly regulate the activity of local ILCs but can also adapt tissue-resident ILC numbers by modulating the viability and proliferative capacity of local ILCs and their potential for transdifferentiation. Moreover, ILCs can be redistributed within an organ or recruited from distal sites to adjust the ILC pool within inflammatory tissue sites according to local requirements. Albeit controversial, homing of human blood ILCs to the site of action represents an interesting, yet underrated phenomenon that requires further analysis (**Figure 1**). Even though enormous progress has been made regarding our knowledge of human ILC regulation, until now, this is largely based on *in vitro* experiments. Human *in vivo* data are, however, mainly restricted to association studies. These have successfully identified great correlations of ILC frequencies with chronic lung and gut inflammation but lack functional evidence. Meanwhile, *in vivo* studies have been primarily conducted on murine ILCs, and translation of functional results to the human system often remains unsatisfying. Therefore, future studies might reinforce the use of humanized mouse models in ILC research. This might allow the central question of whether altered ILC frequencies in disease are cause or consequence to be tackled, which is particularly important with regard to the potential development of ILC-targeting therapeutic strategies.

Until ILCs can be used therapeutically, however, many gaps have to be filled, and our understanding of human ILCs has to be expanded significantly. Therefore, larger patient cohorts should be examined in combination with sophisticated *in vitro* and *in vivo* analyses. Overall, a crucial role of ILCs in mucosal immunity has been impressively determined in the last decade, making the analysis of the functional contribution of human ILCs to fibro-inflammatory diseases and their potential therapeutic modulation a central target for the next 10 years.

AUTHOR CONTRIBUTIONS

AS-K, SW, MN, and IA drafted and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Single-Chain Soluble Receptor Fusion Proteins as Versatile Cytokine Inhibitors

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Cytokines are small secreted proteins that among many functions also play key roles in the orchestration of inflammation in host defense and disease. Over the past years, a large number of biologics have been developed to target cytokines in disease, amongst which soluble receptor fusion proteins have shown some promise in pre-clinical studies. We have previously shown proof-of-concept for the therapeutic targeting of interleukin (IL)-33 in airway inflammation using a newly developed biologic, termed IL-33trap, comprising the ectodomains of the cognate receptor ST2 and the co-receptor IL-1RAcP fused into a single-chain recombinant fusion protein. Here we extend the biophysical and biological characterization of IL-33trap variants, and show that IL-33trap is a stable protein with a monomeric profile both at physiological temperatures and during liquid storage at 4°C. Reducing the N-glycan heterogeneity and complexity of IL-33trap via GlycoDelete engineering neither affects its stability nor its inhibitory activity against IL-33. We also report that IL-33trap specifically targets biologically active IL-33 splice variants. Finally, we document the generation and antagonistic activity of a single-chain IL-4/13trap, which inhibits both IL-4 and IL-13 signaling. Collectively, these results illustrate that single-chain soluble receptor fusion proteins against IL-4, IL-13, and IL-33 are novel biologics that might not only be of interest for research purposes and further interrogation of the role of their target cytokines in physiology and disease, but may also complement monoclonal antibodies for the treatment of allergic and other inflammatory diseases.

Keywords: IL-33, IL-4, IL-13, allergy, inflammation, cytokine, biologics

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INTRODUCTION

Cytokines are small proteins secreted by immune cells that bind to specific high affinity cell surface receptors. This subsequently initiates an intracellular signaling cascade, which culminates in the activation of transcription factors to induce expression of specific genes important for different cellular activities. Cytokines are considered key modulators in host defense against external threats or injury, as well as in initiating and regulating both innate and adaptive immunity. Dysregulated cytokine signaling leads to the development of inflammatory and autoimmune diseases, as well as cancer. Several biologics preventing the activity of cytokines have been developed as new protein-based therapeutics for inflammatory diseases.

These include monoclonal antibodies neutralizing specific cytokines or blocking their receptor, recombinant decoy receptors targeting cytokines, as well as recombinant proteins that can either be cytokine receptor agonists or antagonists (1). Because of their versatility, efficacy and relative ease of large-scale manufacturing, humanized monoclonal antibodies have been the preferred treatment choice and several have emerged as blockbuster drugs. For example, the anti-TNF antibody Adalimumab reduces inflammation in a number of autoimmune diseases including Crohn's disease, ulcerative colitis, plaque psoriasis, rheumatoid arthritis and ankylosing spondylitis (2, 3); Dupilumab is an anti-IL-4Rα monoclonal antibody used to treat allergic diseases (4); and Mepolizumab blocks IL-5 and ameliorates the symptoms of patients suffering from severe eosinophilic asthma (5). On the other hand, recombinant soluble receptor-based cytokine antagonists have also found their way into the clinic. For instance, the dimeric soluble TNFR2 receptor-based immunoglobulin (Ig) G1 Fc fusion protein Etanercept is a blockbuster drug against ankylosing spondylitis, plaque psoriasis, psoriatic arthritis, and rheumatoid arthritis (6). Similarly, Rilonacept is a dimeric fusion protein consisting of the ligand-binding domains of the extracellular portions of the human IL-1 receptor component (IL-1R1) and IL-1 receptor accessory protein (IL-1RAcP) linked to an IgG1 Fc region, which is approved for clinical treatment of cryopyrin-associated periodic syndromes (7, 8). Another cytokine receptor based biologic is Aflibercept, which is a recombinant fusion protein consisting of vascular endothelial growth factor (VEGF)-binding portions from the extracellular domains of human VEGF receptors 1 and 2, that are fused to a human IgG1 Fc portion, and which is used for the treatment of age-related macular degeneration (9). Finally, Anakinra is an example of a recombinant form of the natural anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra) that competes with IL-1 for binding to its receptor, and that was approved for the treatment of rheumatoid arthritis as well as a number of autoinflammatory diseases due to excess IL-1 (10). Despite these clinical successes, existing biologics often only help a subset of patients or suffer from other limitations such as side effects and resistance due to the development of anti-drug antibodies, indicating the need for new or complementary approaches.

IL-33, a member of the IL-1 family of cytokines, is best known for its role in the activation of T helper (Th)2 cell-mediated (also known as type 2) immunity at mucosal body surfaces, where it is released from epithelial and endothelial cells exposed to allergens and other cellular stress factors (11). IL-33 is also considered to function as an "alarmin," activating various immune cells (T cells, macrophages, innate lymphoid cells type 2) upon binding to its cell surface receptor ST2 [reviewed in Braun et al., (11)]. The pathological role of IL-33 is most firmly established in the case of asthma, supported by a large body of experimental data ranging from transgenic overexpression or local intra-tracheal administration of recombinant IL-33, *IL-33* or *ST2* gene ablations, and pharmacological inhibition of the IL-33 signaling pathway in mice (11, 12). Consequently, IL-33-blocking agents are actively developed as new therapeutic biologics. Such agents include anti-IL-33 and anti-ST2 monoclonal antibodies as well as recombinant decoy receptors corresponding to the extracellular part of the IL-33 receptor ST2 (known as soluble ST2 or sST2). For instance, Regeneron Pharmaceuticals, in collaboration with Sanofi, entered Phase 2 clinical trials for asthma, chronic obstructive pulmonary disease and atopic dermatitis with an anti-IL-33 antibody (REGN3500). Another anti-IL-33 monoclonal antibody, Etokimab (AnaptysBio), is also under evaluation or completed Phase2a trials for moderate-to-severe adult atopic dermatitis, chronic rhinosinusitis with nasal polyps, asthma and peanut allergy (13). Moreover, two ST2-targeting monoclonal antibodies, AMG282 (Genentech) and GSK3772847 (formerly CNTO 7160; GlaxoSmithKline), are also in Phase2 clinical trials for asthma.

IL-33 binds with relatively low affinity to its cognate cell surface receptor ST2, which then serves as a binding platform to recruit the co-receptor IL-1RAcP, thus forming a heterodimeric high affinity signaling competent receptor complex (14). This principle led us to engineer a recombinant fusion protein (referred to as "IL-33trap"), comprising the extracellular domains of ST2 (sST2) and IL-1RAcP (sIL-1RAcP) interconnected by a flexible linker, which was anticipated to behave as a high affinity single molecule antagonist of IL-33 cytokine activity. Indeed, IL-33trap showed dramatically enhanced binding affinity to IL-33 when compared to recombinant sST2, which corresponds to the natural decoy receptor for IL-33. Moreover, IL-33trap efficiently prevented the development of airway inflammation and airway hyperreactivity in a murine asthma model (15). More recently, IL-33trap was also shown to suppress colorectal cancer tumor growth by decreasing infiltrating tumor-associated macrophages that negatively impact tumor immunity (16). In the present study, we focus on the further biophysical and biological characterization of the IL-33trap. We also report the generation and characterization of another single chain receptor fusion-based cytokine modulator, termed IL-4/13trap, which exhibits great capacity to inhibit IL-4 and IL-13. Altogether, our data illustrate that single-chain soluble receptor fusion proteins against IL-4, IL-13 and IL-33 are novel biologics that are not only of interest as research tools, but may also complement monoclonal antibodies for the treatment of allergic and other inflammatory diseases.

MATERIALS AND METHODS

Expression Plasmids and Recombinant Proteins

Plasmids have been deposited at the BCCM/GeneCorner plasmid collection (www.genecorner.ugent.be) hosted by our department. p4x-STAT6-Luc2P (LMBP09396), which contains a STAT6-driven luciferase reporter gene, was purchased from Addgene. pNFconluc, which contains an NF-κB–driven luciferase reporter gene, was a gift from Dr. A. Israel (Institut Pasteur, Paris, France), and pACTbgal (LMBP4341) was from Dr. J. Inoue (Institute of Medical Sciences, Tokyo, Japan). Construction of human and mouse IL-33traps, as well as production of mouse IL-33trap in HEK 293 FreeStyle cells, were described previously (15). Full length human IL-33 was PCR amplified

from a human cDNA library and ligated into pCR-Blunt II-TOPO. Splice variants were made by inverse PCR reaction. Subsequently, IL-33 full length and splice variants with a C-terminal 6xHis-tag were PCR amplified and cloned into pJExD by homologous recombination (CloneEZ). The basic bacterial expression vector pJExD, which allows crystal violet-induced expression, was made by modifying the commercial vector pET-Duet1 as follows: lacI and the first T7 promoter and lacO binding site (Eco47III—BamHI) were replaced with a synthetic sequence containing an eilR expression cassette and the crystal violet inducible JExD promoter with eilR binding sites (17). Expression of IL-33 splice variants in *Escherichia coli* BL21 was induced by addition of 100 nmol/L crystal violet (Sigma-Aldrich, Belgium) for 3.5 h at 37°C. Cells were collected by centrifugation, lysed and soluble IL-33 variants were purified using immobilized metal affinity chromatography using Ni-NTA sepharose (IBA Lifesciences, Germany). Production of truncated mouse IL-33 (residues 109–266) in *Escherichia coli* BL21 has been described previously (15). Briefly, protein expression was induced with 1 mmol/L isopropyl β-D-1-thiogalactopyranoside, followed by overnight incubation at 28°C. The bacterial pellet was harvested by means of centrifugation, resolubilized, and lysed by means of sonication. The lysate was centrifuged, and soluble IL-33 was purified from the supernatant by using immobilized metal affinity chromatography, followed by size exclusion chromatography (SEC). Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit.

The ectodomains of the human IL-13Rα1 and IL-4Rα were PCR amplified from a human cDNA library and cloned into pEF6-MycHisA to generate pEF-ShIL13Ra1 and pEF-ShIL4R, respectively. To generate the IL-4/13trap expression plasmid, a human IL-4Rα PCR fragment amplified from pEF-ShIL4R and a linker sequence of 20 times repeating Gly-Gly-Ser (GGS) triplets amplified from pCLG-Duba (LMBP6610) were cloned into the pEF-ShIL13Ra1 vector via 3-way ligation. All constructs were confirmed using DNA sequencing analysis. Human IL-4/13trap and human IL-33trap were produced in HEK293T cells and purified from the medium fraction by immobilized metal affinity chromatography using Ni-NTA sepharose (IBA Lifesciences, Germnay). To reduce the glycosylation complexity and heterogeneity, murine IL-33trap was also produced in suspension growth serum-free adapted HEK293 GlycoDelete cells (18).

Cytokine Bioassays

HEK293T cells (gift from Dr. Hall, Department of Biochemistry, University of Birmingham, United Kingdom) were seeded at 4×10^4 cells/well in 24-well plates and cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS and 2 mmol/L L-glutamine. The next day, cells were transiently transfected by calcium phosphate precipitation with specific IL-33, IL-4, or IL-13 cytokine receptor expression plasmids. For the IL-33 bioassay, cells were co-transfected with the NF-κB reporter plasmid pNFconluc and the constitutively expressing β-galactosidase plasmid pACTbgal. For the IL-4 and IL-13 bioassay, cells were co-transfected with STAT6 and the STAT6 reporter plasmid p4x-STAT6-Luc2P, as well as the constitutively expressing β-galactosidase plasmid pACTbgal. 24 h later, cells were stimulated with recombinant IL-33, IL-4, or IL-13 for 5 h. For cytokine neutralization experiments, cytokines were incubated for 30 min at room temperature with specific cytokine trap inhibitors before addition to the cells. Cell lysates were analyzed for luciferase activity and normalized based on β-galactosidase levels to correct for potential differences in transfection efficiency.

Measurement of Protein Aggregation via SEC-MALLS

Potential protein aggregation was measured via size-exclusion chromatography (SEC) coupled with multi-angle laser light scattering (MALLS). Protein samples were injected onto a Superdex 200 Increase10/300 GL column (GE Healthcare), with PBS pH 7.4 as running buffer at 0.5 ml/min, coupled to an inline ultraviolet-detector (Shimadzu), a multi-angle light scattering miniDAWN TREOS instrument (Wyatt) and an Optilab T-rEXrefractometer (Wyatt) at 25°C. A refractive index increment (dn/dc) value of 0.185 ml/g was used for protein concentration and molecular mass determination. Data were analyzed using the ASTRA6 software (Wyatt). Correction for band broadening was applied using parameters derived from BSA injected under identical running conditions. For the analysis of IL-33traps, conjugate analysis was performed using theoretical protein extinction coefficients and a dn/dc value of 0.160 ml/g for the glycan modifier.

Measurement of Thermostability

Thermostability was measured by ThermoFluor® assay as described (19). Protein samples were diluted in a final volume of 16 µl of PBS buffer and 1 µl of 300X SYPRO Orange (Invitrogen™, Thermo Fisher Scientific) was added. Each experiment was run as a technical triplicate, with a triplicate blank measurement without test protein. Fluorescence in function of the temperature was recorded in a 348-well LightCycler® 480 (Roche Life Science) from 25 to 95°C at 0.02°C/s. Melting temperatures (T_m) were calculated as the V50 value of a Boltzmann sigmoidal curve fitted to the averaged data points of the three replicates in each experiment. Onset temperatures (T_o) were calculated as previously described (20). For graphing, the raw data sets were averaged, blank corrected and then normalized (minimal value at 0%, maximal value at 100%), using Prism 7 Software (GraphPad).

RESULTS

Use of a Flexible Linker Allows the Generation of an Fc-Less Single-Chain IL-33trap

Recombinant cytokine decoy receptor-based biologics such as Etanercept, Rilonacept, and Aflibercept all contain the Fc portion of human IgG1. Fusion to an Fc domain allows dimerisation of the two receptor subunits and provides manufacturing, biological and pharmacological advantages *in vivo*, including established

large-scale affinity purification, half-life extension due to pH-dependent binding to the neonatal Fc receptor (FcRn) (21). For the construction of IL-33trap we used an alternative approach that avoids the need for an Fc to induce receptor dimerisation. More specifically, we cloned a flexible GGS linker sequence between sST2 and sIL-1RAcP to ensure the intramolecular formation of an active sST2/sIL-1RAcP heterodimeric receptor complex (15). Although at that time we did not compare the bioactivity of our linker containing IL-33trap with an IL-33trap variant that does not contain a linker, it is likely that the linker is needed for optimal domain orientation and heterodimerisation between both receptor subunits and consequently the bioactivity of IL-33trap. To further compare the effect of a linker sequence with the effect of fusion to an Fc-moiety on IL-33trap bioactivity, we generated recombinant human IL-33trap variants in which both receptor subunits (sST2 and sIL-1RAcP) were either not separated by a linker or contained a 7x or 12x GGS linker (shown empirically to enable the formation of biologically active IL-33trap), and compared these with the corresponding IL-33trap constructs that also contain a human IgG1 Fc domain at the C-terminus (**Figure 1A**).

The antagonistic activity of different human IL-33trap constructs was analyzed by measuring their ability to inhibit IL-33-induced activation of an NF-κB-dependent luciferase reporter gene in HEK293T cells that were made IL-33 responsive by transient transfection with human ST2. Prior to cell stimulation, recombinant human IL-33 was incubated for 30 min with equimolar concentrations of each human IL-33trap variant over a range of inhibitors/target ratios. An IL-33trap without Fc and GGS linker had an almost 10-fold reduced activity compared to a similar GGS-less construct fused to an Fc (IC_{50} of 748 pM and 90 pM, respectively; **Figure 1B**), suggesting that the Fc-moiety allows the optimal IL-33trap conformation for ligand binding, most likely by mediating dimerisation of two IL-33trap molecules. Importantly, inclusion of a 7x GGS or 12xGGS linker sequence instead of an Fc also enhanced the antagonistic effect of the IL-33trap (IC_{50} of 194 pM and 42 pM, respectively; **Figure 1C**), with sIL-1RAcP-12xGGS-sST2 being slightly more potent than sIL-1RAcP-sST2-Fc (IC_{50} of 42 vs. 90 pM; **Figure 1C**). Combined use of a 12xGGS linker and an Fc fusion in a single IL-33trap construct (IC_{50} of 57 pM) did not much further change the potency of a 12xGGS-only or Fc-only construct (IC_{50} of 42 or 90 pM, respectively; **Figures 1C,D**). However, additional Fc fusion leads to a more potent molecule in the case of a shorter 7xGGS linker (IC50 67 vs. 194 pM; **Figures 1C,D**). Collectively, these data illustrate that fusion of either a GGS linker sequence or an Fc-moiety increases the IL-33 antagonistic activity of an sIL-1RAcP-sST2 fusion protein by enabling, respectively, intramolecular and intermolecular interactions between sST2 and sIL-1RAcP that are necessary for the formation of a high affinity IL-33 binding complex. The lower potency of a 7xGGS containing construct (IC_{50} of 194 pM) compared to a 12xGGS (42 pM) construct suggests that a longer linker increases molecular flexibility, favoring a more optimal intramolecular interaction between sST2 and sIL-1RAcP. Importantly, our data illustrate that insertion of a flexible 12xGGS linker between both IL-33 receptor subunits allows the formation of a single-chain fusion protein with high IL-33 antagonistic activity, circumventing the need for Fc fusion and Fc-mediated dimerisation.

IL-33trap Is Biophysically Stable During Liquid Storage and at Physiological Temperature

The biophysical behavior of therapeutic proteins is very important for the correct development and optimisation of biologics, since it may impact many aspects of drug function, stability and activity. Excessive aggregates and fragmentation, as well as denaturation or oxidation are indicative of an unstable product, unsuitable for *in vivo* use. For this reason, we sought to characterize the molecular and thermal stability of the murine 20xGGS-linker containing but Fc-less IL-33trap for which we previously showed an inhibitory effect upon local delivery in a mouse asthma model (15). First, we examined whether IL-33trap is prone to form aggregates during storage. IL-33trap was stored frozen in PBS at −80°C or liquid-stored at 4°C for a time period of 10 days. To determine the presence of potential aggregates, we used size-exclusion chromatography (SEC) coupled with ultraviolet (UV), multi-angle light scattering laser (MALLS) and refractive index (RI) detectors. Protein elution fractions were identified as eluting species with a peak in both UV absorbance and differential RI (dRI) intensity. IL-33trap, either frozen-stored at −80°C or liquid-stored at 4°C for 10 days, was found to be highly homogeneous, adopting monodisperse assemblies, with a protein molecular mass comparable to the theoretical (**Figure 2A**).

We next assessed the thermostability of IL-33trap using ThermoFluor® assay (19), and melting temperature (T_m) was calculated as the V50 value of a Boltzmann sigmoidal curve fitted to the melting curve (19, 22). The analysis showed a T_m and a T_o of 55 and 46.5°C, respectively which is well above the physiological temperature of 37°C (**Figure 2B**). Together, our data indicate that IL-33trap is a stable molecule at physiological temperature and does not form aggregates when liquid-stored at 4°C for up to 10 days.

Glycosylation Does Not Affect IL-33trap Bioactivity

We previously showed that mouse IL-33trap expressed in HEK293T cells is glycosylated (15). To further estimate the degree of glycosylation, we calculated the total protein and glycan molecular weight on IL-33trap using SEC-MALLS and ASTRA6 software (Wyatt), showing that IL-33trap is heavily glycosylated (25–35% of total mass) (**Figure 2A**). Using NetNglyc 1.0 Server and NetOglyc 4.0 Server prediction (23), 15 potential N-glycosylation and 1–4 potential O-glycosylation sites were found. Although not all of these sites are likely to be occupied, the high number of glycosylation sites leads to significant product heterogeneity, which is further complicated by the inherent difference in glycan chain length and complexity in eukaryotic expression systems. This is a major challenge for efficient purification at high yield, to set sound specifications for product release and hence to assure batch reproducibility. It is also

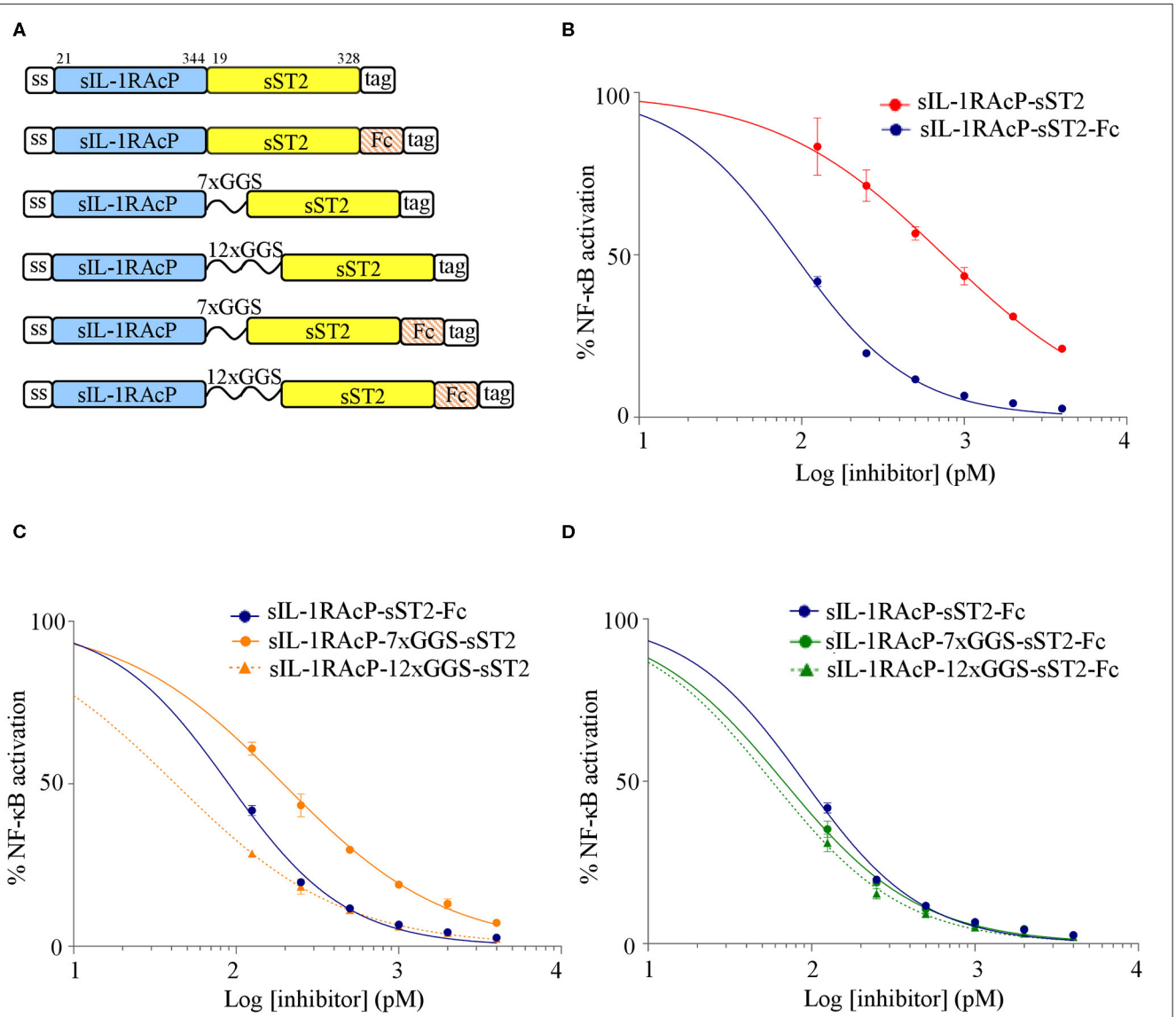

FIGURE 1 | Comparison of the effect of Fc fusion and introduction of a 12xGGS linker on the inhibitory capacity of human IL-33trap. **(A)** Schematic representation of human IL-33trap constructs (numbers indicate the amino acid boundaries of the receptor ectodomains). **(B–D)** Effect of different fusion proteins on IL-33 induced NF-κB activation. HEK293T cells were treated with recombinant human IL-33 that was pre-incubated with equimolar concentrations of the indicated fusion proteins over a range of inhibitors/target ratios and assayed for NF-κB activity as described in Material and Methods. Values represent means ± SE of technical triplicates. Results are representative of at least two independent experiments.

likely to attribute significant heterogeneity in pharmacokinetic behavior of the molecule, due to differential lectin-mediated blood clearance of different glycoforms. Therefore, we decided to reduce the glycosylation complexity and heterogeneity of mouse IL-33trap using HEK293 GlycoDelete technology (18), which reduces glycosylation to short single-branch oligosaccharides that are partially sialylated. Indeed, IL-33trap produced in HEK293 GlycoDelete is notably less glycosylated (6% of the total mass) compared to the original IL-33trap molecule produced in HEK293 FreeStyle cells (25–35% of the total mass) (**Figure 2A**). We then compared the thermostability and antagonistic activity of the glycosylated and under-glycosylated IL-33trap variants. Reduced glycosylation had only a mild effect on the melting temperature (**Figure 2B**), and did not affect the ability of IL-33trap to inhibit IL-33-induced NF-κB activation (**Figure 2C**).

IL-33trap Specifically Targets Active IL-33 Splice Variants

Human IL-33 mRNA can be alternatively spliced in several smaller IL-33 splice variants lacking exon 3, 4, 5 or a combination thereof, which are expressed in different cell types in different proportions (24). In contrast to deletion of exons 3 and 4, absence of exon 5 results in loss of IL-33 activity (25), which is consistent with the fact that exon 5 encodes amino acid residues

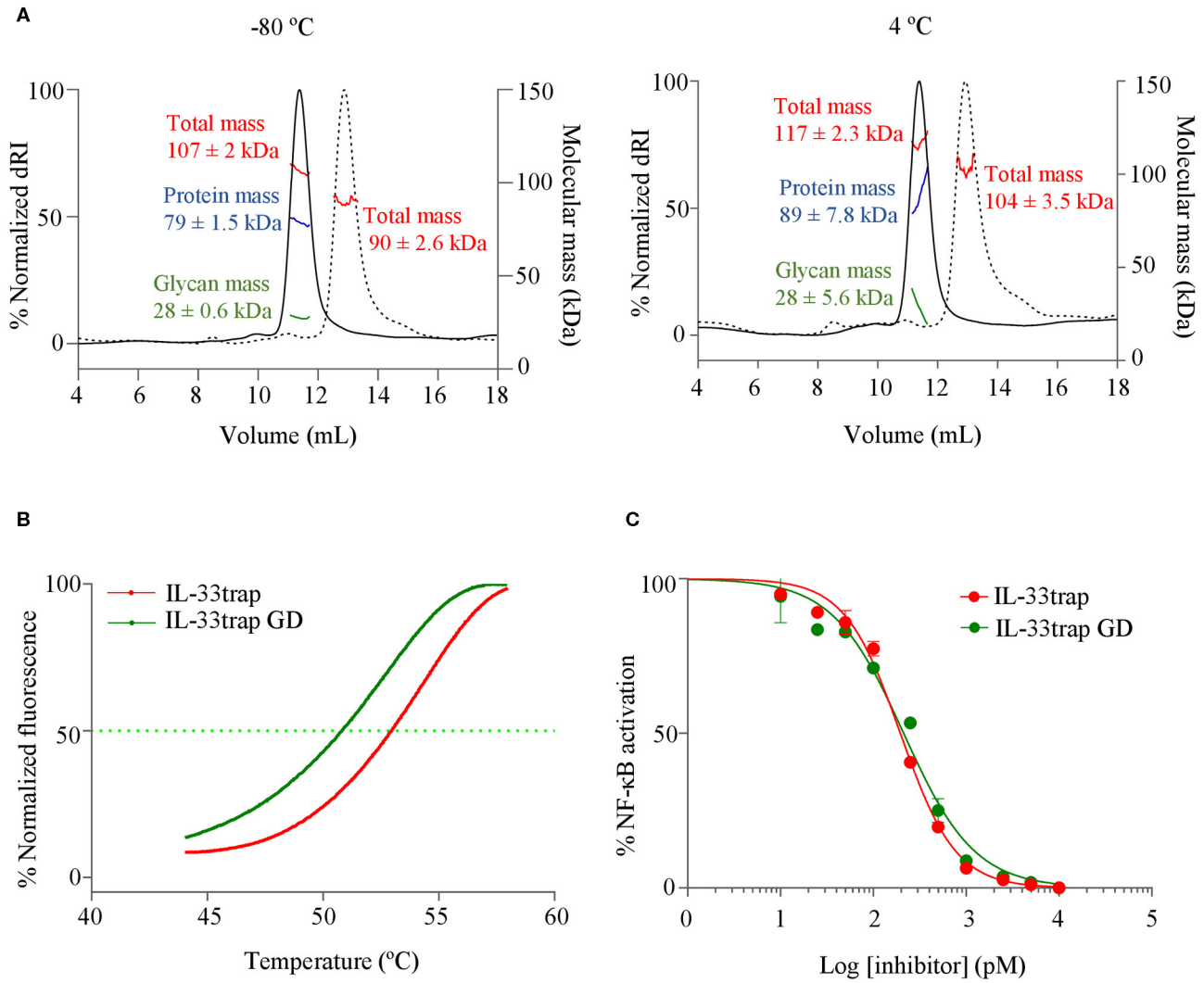

FIGURE 2 | Glycosylated and under-glycosylated murine IL-33trap do not form aggregates and are biophysically stable during liquid storage and at physiological temperature. **(A)** SEC-MALLS analysis of murine 20xGGS linker-containing IL-33trap expressed in HEK293T or HEK293 GlycoDelete cells (referred as IL-33trapGD) and frozen-stored vs. liquid-stored at 4°C for a time period of 10 days. SEC elution profiles of IL-33trap (full line) and IL-33trapGD (dashed line) are plotted as the normalized differential refractive index (dRI, left vertical axis) as a function of the elution volume (mL). Molecular weight of IL-33trap and IL-33trapGD as determined by MALLS (kDa, right vertical axis) is dissected by protein conjugate analysis in total (red), protein (blue) and sugar (green) mass and is reported as the number-average molar mass across the elution peak ± SD. Only the total mass (red) of IL-33trapGD is reported as protein conjugate analysis did not yield significant glycan mass. *n* = 1 sample injected. SEC-MALLS data was analyzed using ASTRA6 software (Wyatt) and graphs were plotted using GraphPad Prism software. **(B)** Melting curves of murine IL-33trap and IL-33trapGD plotted as normalized fluorescence intensity in function of increasing temperature, determined by ThermoFluor® assay. Values of melting temperature (T_m) and onset temperature (T_o) were calculated using GraphPad Prism software. **(C)** Comparison of the effect of IL-33trap and IL-33trapGD on IL-33 induced NF-κB activation. HEK293T cells were treated with recombinant murine IL-33 that was pre-incubated with murine IL-33trap or IL-33trapGD and assayed for NF-κB activity as described in Material and Methods. Values represent means ± SE of technical triplicates. Results are representative of at least two independent experiments.

that are critical for ST2 binding (26). Importantly, binding of inactive IL-33 isoforms to IL-33-neutralizing biologics would serve as a natural sink and decrease drug availability. However, because IL-33trap is a receptor-based biologic, it is expected to exclusively interact with bioactive receptor-binding IL-33 isoforms and to be insensitive to the presence of inactive IL-33 splice variants. To further test this hypothesis, we generated IL-33 splice variants lacking exons 3 and 4 (IL-33Δe3-4) or exons 3, 4 and 5 (IL-33Δe3-5) (**Figure 3A**), and analyzed their activity in an IL-33 bioassay. As shown previously, IL-33Δe3-4 efficiently induced NF-κB activation, while IL-33Δe3-5 was inactive (**Figure 3B**). Furthermore, NF-κB activation induced by IL-33Δe3-4 was efficiently inhibited by IL-33trap (**Figure 3C**). We next performed a competition assay, where we

pre-incubated mature IL-33 with IL-33trap in the presence of increasing concentrations of either IL-33Δe3-4 or IL-33Δe3-5 splice variants. Consistent with its receptor-binding capacity, addition of IL-33Δe3-4 reduced the ability of IL-33trap to inhibit IL-33 signaling, while addition of IL-33Δe3-5 had no effect (**Figure 3D**). These results illustrate that inactive IL-33 splice variants will not act as a sink for IL-33trap, which might offer a significant advantage compared to certain monoclonal antibodies that, depending on the recognized epitope, might not always distinguish between active and inactive IL-33 isoforms.

Generation and Validation of a Human IL-4/13 Trap as a Dual Cytokine Antagonist

As for the IL-33trap, a similar approach using single-chain soluble receptor fusion proteins can be used to target other cytokines that signal through a heterodimeric receptor complex. In this regard, we have previously reported the design and validation of TSLP-trap, a fusion protein which consists of the extracellular domains of TSLPR and IL-7Rα fused via a 20xGGS flexible linker (27). Together with IL-33 and IL-25, the epithelium-derived cytokine TSLP is considered a central orchestrator of Th2 responses in atopic disorders, and therefore a promising therapeutic target. Similarly, IL-4 and IL-13 are in the spotlight as interesting dual therapeutic targets in type 2-driven inflammatory disease (28). Importantly, these two cytokines both bind to the type II receptor consisting of IL-4 receptor alpha (IL-4Rα) and IL-13Rα1 (29) (**Figure 4A**), offering a possibility to simultaneously target two cytokines with one inhibitor. Using a similar design as IL-33trap (15), we generated and validated a new human IL-4/13trap, consisting of the extracellular domain of human IL-13Rα1 fused to the extracellular domain of human IL-4Rα via a flexible 20xGGS linker. The expression construct also contains the human IL-13Rα1 signal sequence at the N-terminus, which allows protein secretion, and a myc/His tag at the C-terminus to facilitate protein purification and detection (**Figure 4A**). Human IL-4/13trap was produced using HEK293 FreeStyle cells to ensure proper folding, and purified from conditioned media using immobilized metal affinity chromatography and size exclusion chromatography, as described in materials and methods.

To test whether IL-4/13trap displays antagonistic properties, we investigated its ability to inhibit both IL-4 and IL-13 signaling in a cell-based assay. To this end, HEK293T cells were made responsive to either IL-4 or IL-13 by transfection with human IL-4Rα and IL-13Rα1, and downstream STAT6 activation was followed via STAT6-dependent luciferase reporter gene expression. IL-4 as well as IL-13 treatment resulted in robust STAT6 activation (**Figure 4B**). Importantly, pre-incubation of either IL-4 or IL-13 with equimolar concentration of IL-4/13trap strongly reduced IL-4- and IL-13-induced STAT6-dependent luciferase activity (IC_{50} of 465 pM and 434 pM, respectively) (**Figure 4B**). These data convincingly demonstrate that IL-4/13trap behaves as a strong IL-4 and IL-13 antagonist and illustrate the feasibility to develop recombinant single-chain soluble receptor fusion proteins as novel biologics for the inhibition of a wide range of cytokines or other protein ligands that signal via a heterodimeric receptor complex.

DISCUSSION

Because of the important role of cytokines in human autoimmune and inflammatory diseases, many biologics targeting cytokines and their receptors have been developed over the past years. Several soluble receptor-based biologics, such as the TNF antagonist Etanercept, the IL-1 antagonist Rilonacept, and the VEGF antagonist Aflibercept, are already actively used in the clinic as alternatives for monoclonal antibodies. In all cases, soluble receptors were engineered to encode an IgG Fc region to increase half-life and to permit dimerisation and high affinity ligand binding. A similar approach has been described for IL-4 and IL-6 neutralizing trap molecules (30), which have not yet entered the clinic. The use of Fc fusion to enable dimerisation doubles the size of the inhibitor (> 250 kDa in the case of Rilonacept, glycosylation not included), limiting its tissue permeability, which may be even more critical in certain conditions such as asthma where mucus imposes an additional barrier. Moreover, larger proteins typically result in lower expression levels while higher dosing is needed compared to smaller proteins (∼90 kDa in the case of IL-33trap) to achieve the same molar concentration. The presence of an Fc portion could also lead to side effects due to nonspecific binding to Fc receptors or Fc-associated effector functions, although Fc engineering can also overcome such problems. However, it remained unclear whether Fc-fusion-driven bivalency also contributes to the cytokine neutralizing activity of soluble receptor fusion proteins like the IL-33trap. We have demonstrated here that the Fc moiety strongly enhances the inhibitory capacity of a linker-less IL-33trap molecule, indicating a role for Fc-mediated dimerisation in the formation of a fully functional soluble receptor complex. The need for Fc-mediated dimerisation could however be completely replaced by the introduction of a flexible linker (12xGGS) between both receptor subunits, which allowed the formation of a single-chain fusion protein with an optimal conformation for high affinity ligand binding. The use of a flexible linker in the design of IL-33trap thus offers a significantly different candidate biotherapeutic as an alternative to Fc fusion. The use of Fc-less fusion biologics could be especially relevant for maximizing exposure of the therapeutic in certain tissues like the eye and lung upon local delivery, where FcRn-mediated transcytosis of Fc-fusion proteins across epithelial and endothelial cells would otherwise mediate transport into systemic circulation.

The manufacturability of therapeutic proteins entails major challenges to ensure drug efficacy and safety. Therefore, appropriate molecular characterization and optimisation of these biologics is crucial to ensure protein stability, homogeneity, low immunogenicity as well as optimal pharmacokinetic properties. Therapeutic proteins are often susceptible to thermal stress, which can cause drastic conformational changes resulting in reduced drug efficacy and stability. Furthermore, protein unfolding *in vivo* may favor intermolecular protein interactions

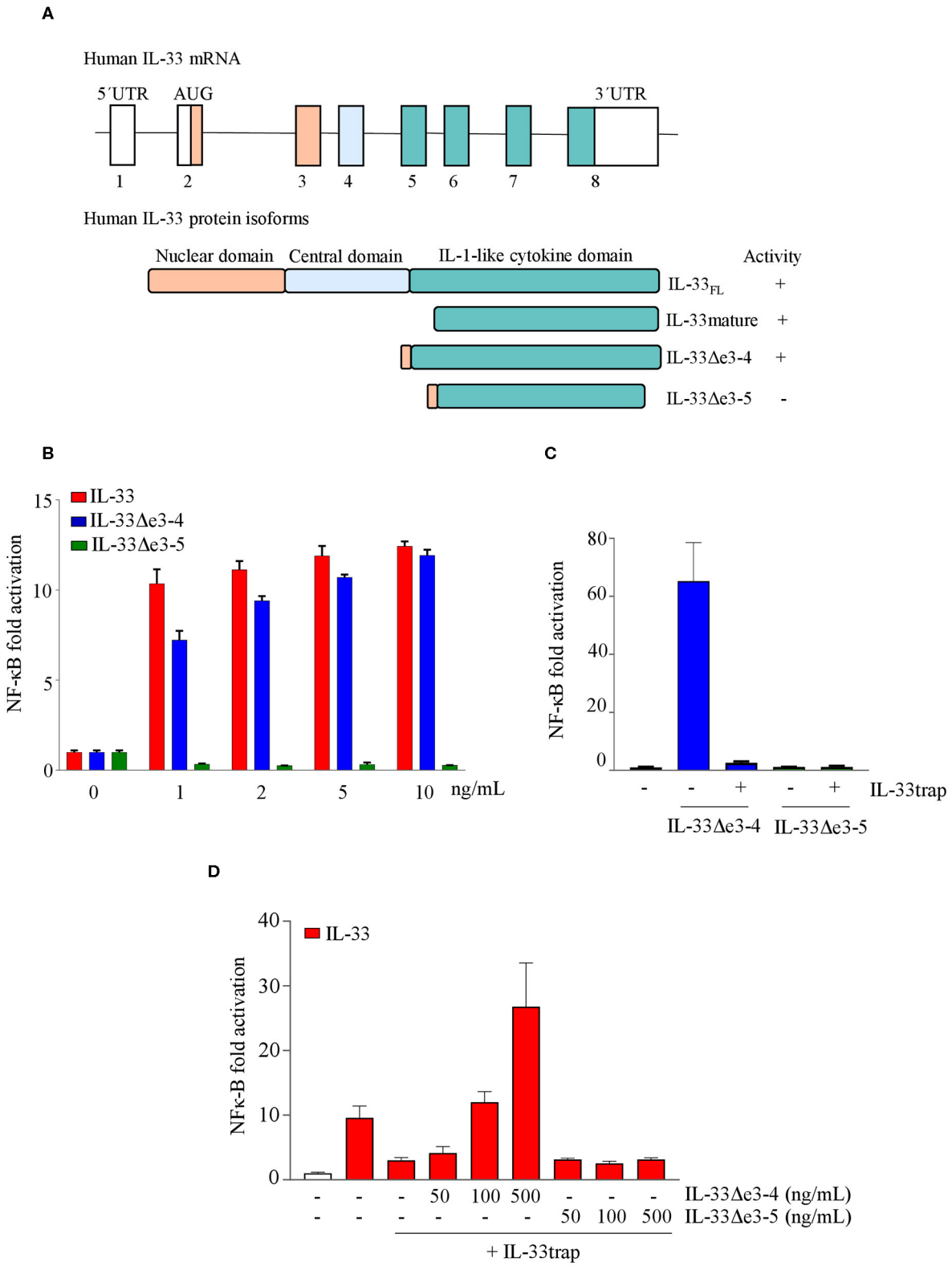

FIGURE 3 | Human IL-33trap specifically targets active IL-33 splice variants **(A)** Schematic representation of human IL-33 splice variants and encoded protein isoforms. **(B)** NF-κB activation induced by different IL-33 splice variants. **(C)** Effect of IL-33trap on NF-κB activation induced by different IL-33 splice variants. **(D)** Effect of the presence of IL-33 splice variants on the ability of IL-33trap to inhibit IL-33 induced NF-κB activation. NF-κB activity was measured in HEK293T cells as described in Material and Methods. Values represent means ± SE of technical triplicates. Results are representative of at least two independent experiments.

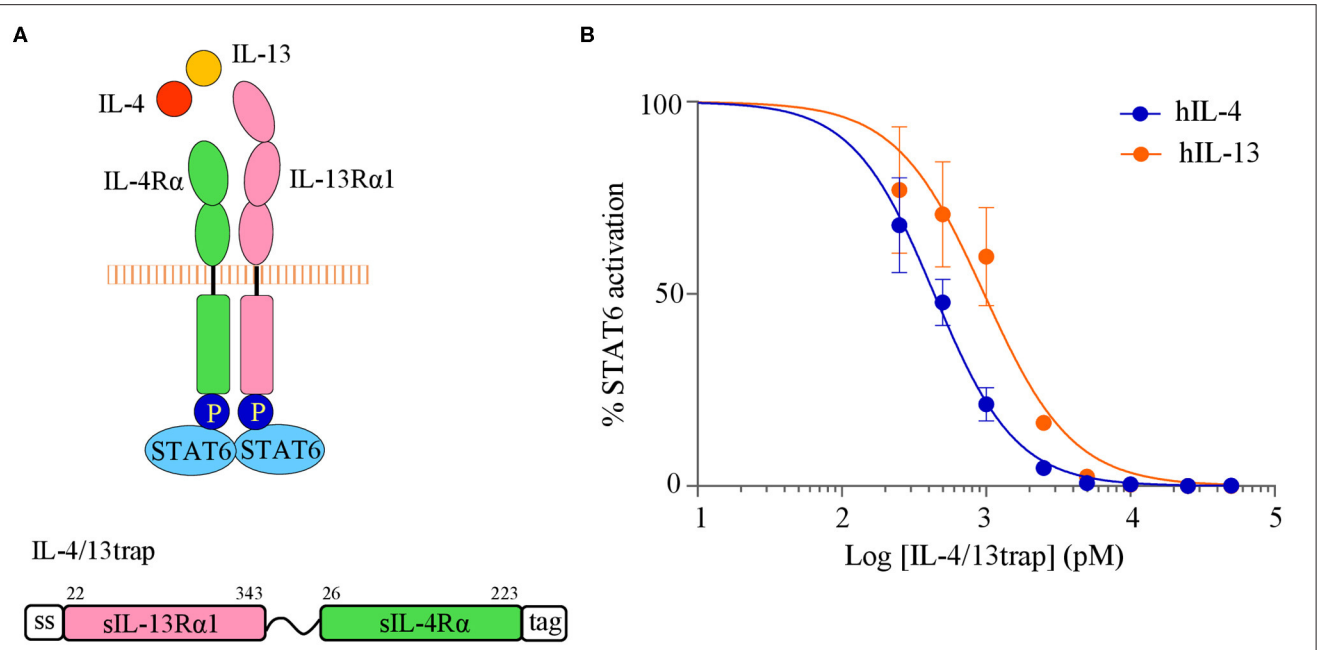

FIGURE 4 | Human IL-4/13trap inhibits IL-4 and IL-13 activity. **(A)** Schematic representation of the type II receptor complex for IL-4 and IL-13 (also receptor phosphorylation and recruitment of STAT6 is shown), and the corresponding human IL-4/13trap construct (numbers indicate the amino acid boundaries of the receptor ectodomains). **(B)** Effect of human IL-4/13trap on IL-4- and IL-13-induced STAT6 activation. HEK293T cells were treated with recombinant human IL-4 or IL-13 that was pre-incubated with human IL-4/13trap and assayed for STAT6 activity as described in Material and Methods. Values represent means ± SE of technical triplicates. Results are representative of at least two independent experiments.

and subsequent aggregates formation, which in turn could trigger immune responses inducing formation of anti-drug antibodies (31, 32). SEC-MALLS analysis indicated that IL-33trap does neither form aggregates nor undergo denaturation above physiological temperatures. Moreover, IL-33trap stability was not affected during liquid storage, which may be of interest for clinical practices as it might facilitate patient self-administration. The quality of protein-based biologics may also be determined by protein glycosylation. Inherent differences in glycan chain length and complexity in eukaryotic expression systems can result in highly heterogeneous and complex glycosylation pattern (33). This is a major challenge to ensure efficient purification at high yield and batch reproducibility. Our data show that reducing glycosylation complexity and heterogeneity of IL-33trap using HEK293 GlycoDelete technology does not affect its bioactivity.

The complexity of cytokine networks is drastically increased by the generation of multiple cytokine and cytokine receptor isoforms due to alternative splicing, differential promotor usage, or posttranslational modifications such as proteolytic cleavage and degradation (34, 35). This increases the risk that certain active isoforms or variants may escape recognition by epitope-specific monoclonal antibodies, leading to drug resistance. Unfortunately, the specific epitopes that are recognized by monoclonal antibodies that are in clinical development have not been reported in literature. Alternatively, inactive isoforms that do bind monoclonal antibodies may act as a sink, again reducing that efficacy of the biologic. Also in the case of IL-33, alternative splicing (24) and proteolytic cleavage (34) have been described, creating tens of active and inactive IL-33 isoforms. Possibly, some of these variants may escape or interfere with currently used IL-33 neutralizing monoclonal antibodies, which may lead to poor responses in preclinical or clinical studies. Importantly, as the here described soluble receptor-based cytokine traps fully mimic cytokine-binding by endogenous cell surface receptors, they are expected to neutralize all biologically active cytokine isoforms and be insensitive to the presence of inactive isoforms, as also documented in the present study in the case of specific IL-33 splice variants.

Although targeting of specific cytokines with receptor-based fusion proteins and monoclonal antibodies have demonstrated beneficial clinical outcomes in patients suffering from a wide variety of inflammatory disorders (1), functional redundancy of cytokines as well as the development of anti-drug antibodies often limits the success of specific cytokine therapies. Thus, combinatorial treatment approaches simultaneously targeting several cytokines may improve clinical outcomes, which has also been documented for IL-33 and TSLP in preclinical studies (36). Therefore, combination of IL-33trap with TSLPtrap, which we have previously described as a 20–30 fold more potent TSLP inhibitor *in vitro* than the anti-TSLP antibody Tezepelumab (27), might be an interesting therapeutic approach. Dual targeting of IL-4 and IL-13 with Dupilumab, a monoclonal antibody targeting IL-4Rα, has recently entered the clinic for certain allergic diseases (37, 38). Likewise, a novel bispecific llama-based antibody simultaneously targeting IL-4Rα and IL-5, providing a triple blockade of IL-4, IL-13 and IL-5 signaling, has been

developed (39). Of interest, simultaneous inhibition of IL-13 and IL-33 signaling was shown to inhibit allergic airway inflammation in mice more effectively than inhibition of either cytokine alone (40). In the present study we have shown that our design of single-chain soluble receptor fusion proteins for the development of cytokine traps is not only applicable to IL-33 and TSLP, but also to IL-4 and IL-13. It will therefore be of interest to further test the effect of the here described IL-4/13trap in preclinical mouse models. Also, the generation of multi-specific cytokine trap therapeutics, by fusing different cytokine trap proteins, might be a path worth to consider. So far, only murine IL-33trap has been tested *in vivo* in a mouse asthma model (15). It will be interesting to also develop clinically relevant *in vivo* models where the human IL-33 trap can be tested. In preliminary experiments we have observed that repetitive intratracheal administration of human IL-33 in mice induces lung eosinophilia, which can be prevented by treatment of mice with human IL-33trap. Use of humanized mice reconstituted with human immune cells (41), which are then intratracheally injected with human IL-33, might also be an option. Ideally, one might use humanized mice in which the murine *IL-33* gene has been replaced by the human *IL-33* gene, which would allow to test the effect of human IL-33trap in an allergic disease mouse model that relies on human IL-33 production from epithelial or endothelial cells. However, it remains to be seen if such a human IL-33 transgene will be regulated similarly to the mouse gene in a murine context. Similar approaches might be applicable for preclinical studies using IL-4/13trap.

In conclusion, following the quest for additional novel anti-cytokine biologics, our data illustrate the potential of recombinant single-chain soluble receptor fusion proteins as novel anti-cytokine biologics. Cytokine traps targeting IL-33, TSLP, IL-4 and IL-13 are novel tools that nicely complement the use of monoclonal antibodies for the treatment of allergic diseases. Moreover, the translational impact of such therapeutics can be expected to be much broader than allergic diseases.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AH, HB, and IA designed the experiments. AH, HB, DV, and IA performed experiments. NC provided the HEK293 GlycoDelete cells and associated protein production protocols. SS and KV helped with the biophysical characterization of the IL-33trap. AH, IA, and RB wrote the manuscript. RB and IA supervised the work. All authors contributed to the scientific discussion.

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REVIEW
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Structural Understanding of Interleukin 6 Family Cytokine Signaling and Targeted Therapies: Focus on Interleukin 11

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Cytokines are small signaling proteins that have central roles in inflammation and cell survival. In the half-century since the discovery of the first cytokines, the interferons, over fifty cytokines have been identified. Amongst these is interleukin (IL)-6, the first and prototypical member of the IL-6 family of cytokines, nearly all of which utilize the common signaling receptor, gp130. In the last decade, there have been numerous advances in our understanding of the structural mechanisms of IL-6 family signaling, particularly for IL-6 itself. However, our understanding of the detailed structural mechanisms underlying signaling by most IL-6 family members remains limited. With the emergence of new roles for IL-6 family cytokines in disease and, in particular, roles of IL-11 in cardiovascular disease, lung disease, and cancer, there is an emerging need to develop therapeutics that can progress to clinical use. Here we outline our current knowledge of the structural mechanism of signaling by the IL-6 family of cytokines. We discuss how this knowledge allows us to understand the mechanism of action of currently available inhibitors targeting IL-6 family cytokine signaling, and most importantly how it allows for improved opportunities to pharmacologically disrupt cytokine signaling. We focus specifically on the need to develop and understand inhibitors that disrupt IL-11 signaling.

Keywords: cytokine, interleukin, IL-11, IL-6, JAK, STAT, structural biology, drug development

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INTRODUCTION

Cytokine Signaling—A Brief History

In 1957, interferons were the first cytokines to be identified as secreted protein products induced following virus infection (1). In the subsequent decades, similar proteins, including the colony stimulating factors (CSFs) (2–4), Interleukin (IL)-2 (5, 6), and IL-3 (7, 8) were identified as secreted molecules able to support the growth of various hematopoietic cell linages *in vitro*. In 1974, the broad term "cytokine" was introduced (9) and in 1979 the term "interleukin" was introduced to standardize the names of the proteins now known as IL-1 and IL-2 (10). Over the next decade, radiolabelling studies revealed that cytokines bound distinct and unique receptors on the cell surface (11). It was also revealed that some cytokines, such as granulocyte-macrophage CSF (GM-CSF), IL-5 and IL-3 compete for a low-affinity receptor (12, 13), foreshadowing the identification of the β common receptor.

Following the discovery of the first cytokines, the mechanisms of intracellular signal transduction by cytokines remained elusive. The first transcriptional activator to be well-characterized was interferon-stimulated gene factor 3 (ISGF3), a multi-component protein complex consisting of what is now known as signal transducer and activator of transcription (STAT)1 and STAT2 (14, 15). Subsequently, related STAT proteins were identified as being activated *via* cytokine stimulation (16, 17). It was also shown that these factors were tyrosine phosphorylated (18, 19) on cytokine activation. The kinases responsible for this phosphorylation, the Janus kinases (JAKs) were first identified through a PCR screen of a murine hematopoietic cell line (20, 21). Their significance was unclear until the early 1990s, when they were shown to be activated as a result of cytokine binding and to phosphorylate the transcription factors that were already identified as key for interferon signal transduction (22). Subsequently, different members of the JAK family were found to be responsible for signal transduction by numerous cytokines (23–25). In 1997, the negative feedback regulators of the pathway, the suppressors of cytokine signaling (SOCS) proteins were identified (26–28). The key components of cytokine signaling using the JAK-STAT pathway were thus understood by the late 1990s, although many of the detailed molecular mechanisms are still unknown and remain under intense investigation today.

IL-6 family cytokines belong to a large group that signal *via* the JAK-STAT pathway, are characterized by a four α-helical bundle structure, and share receptors with similar structures consisting of several fibronectin type III (Fn3) and immunoglobulin-like (Ig-like) domains (29–31). Other cytokines, such as the IL-1/IL-18 family and the TNF-α family are structurally distinct from the four-α helical bundle family (32), utilize different signaling mechanisms, and are thus beyond the scope of this review. Conversely, several protein hormones, such as leptin, growth hormone (GH), prolactin and erythropoietin (EPO) utilize similar signal transduction mechanisms, are structurally related to the four-α helical bundle cytokines, and are thus best categorized alongside them (30, 33). The discovery of GH and EPO predate that of the interferons by several decades (34–37), but they were not recognized as related until they were cloned, sequenced, and significant sequence homology was noted between the receptors, GHR and EPOR (38, 39).

The Structure of Cytokines and Their Receptors

The four-α helical bundle cytokine family is the largest cytokine family. Both class I cytokines (e.g., GH, IL-6, IL-11) and class II cytokines (e.g., IFN-α, IL-10) utilize receptors that are broadly similar in structure and initiate similar intracellular signaling mechanisms (29). Cytokines from both classes are characterized by a compact α-helical bundle formed by four anti-parallel α-helices, arranged in an up-up-down-down topology (29, 31). This arrangement of helices necessitates long loops joining the helices (**Figure 1A**). Secondary structure in the loops is common, for example, the loop joining the C and D helices in IL-6 (the CD loop) contains a short α-helix (45), and in IL-4 (46) and GM-CSF (41), the AB and CD loops form a small anti-parallel β-sheet on the same face of the cytokine (**Figure 1A**). The topology of the four-α helical bundle fold provides a large surface area for the cytokine to bind its receptors.

Cytokine receptors are generally modular, single-pass transmembrane proteins, with a large extracellular region consisting of multiple all-β Ig-like domains and Fn3 domains (33). Both domains possess a β-sandwich structure, with two anti-parallel β sheets (**Figure 1B**). The exception are the IL-2Rα/IL-15Rα receptors, which consist of two all-β sushi domains, unrelated to the Ig and Fn3 domains comprising other cytokine receptors (33, 47, 48). The cytokine binding domains of the receptors consist of two Fn3 domains at approximately a 90° angle, forming the cytokine binding homology region (CHR) (30). Cytokines bind at the junction of these two domains. Each of the two domains of the CHR possess conserved features, the N-terminal domain of the CHR has two conserved disulphide bonds, and in class I cytokine receptors of the C-terminal domain of the CHR has a highly conserved Trp-Ser-X-Trp-Ser motif (WSXWS) motif (30). The WSXWS motif generally forms a "ladder" consisting of cation-π interactions between the tryptophan and arginine side chains. The precise structural role of the WSXWS motif is still unclear. It may stabilize the receptor, since mutations in the WSXWS motif result in a non-functional receptor (49, 50), and a rare genetic disease results from a mutation in the WSXWS motif of GHR (51). In IL-21Rα, the first Trp of the WSXWS motif is C-mannosylated and this modified Trp forms stabilizing interactions with other glycans and amino acid residues in the structure (52). The extensive glycosylation, both Trp C-mannosylation, and N-linked glycosylation gives IL-21Rα the structure of an "A-frame," with a glycan chain forming a bridge between the two domains in the receptor. Similar Trp C-mannosylation has been detected in the p40 subunit of IL-12 by mass spectrometry (53), but has not been observed in crystal structures which include p40 (54–56), possibly reflecting incomplete incorporation of the modification in recombinant protein. Recent studies have suggested that, in addition to being a stabilizing structural element, the WSXWS motif undergoes a conformational change on cytokine binding, suggesting it has a role in receptor activation (57).

Beyond the CHR, many cytokine receptors have additional extracellular domains. These domains have varied roles, for example in correctly orienting the receptor to allow the activation of intracellular kinases (58), to facilitate cytokine binding (59), or to modulate intracellular trafficking to the membrane (60). While, most cytokine receptors are single-pass transmembrane proteins, an exception is the ciliary neurotrophic factor (CNTF) receptor, which is lipid anchored (61). The structures of cytokine receptor transmembrane domains have been solved, generally by nuclear magnetic resonance (NMR) spectroscopy (62–64). Single-pass transmembrane cytokine receptors also possess an intracellular domain that is assumed to be highly dynamic (65, 66). In the case of signal-transducing cytokine receptors, the intracellular domain binds signal transducing molecules, such as the JAKs, STATs, and the SOCS proteins.

Understanding the molecular details of cytokine engagement requires detailed structural knowledge of the complexes formed by cytokines and receptors. The first cytokine/receptor complex

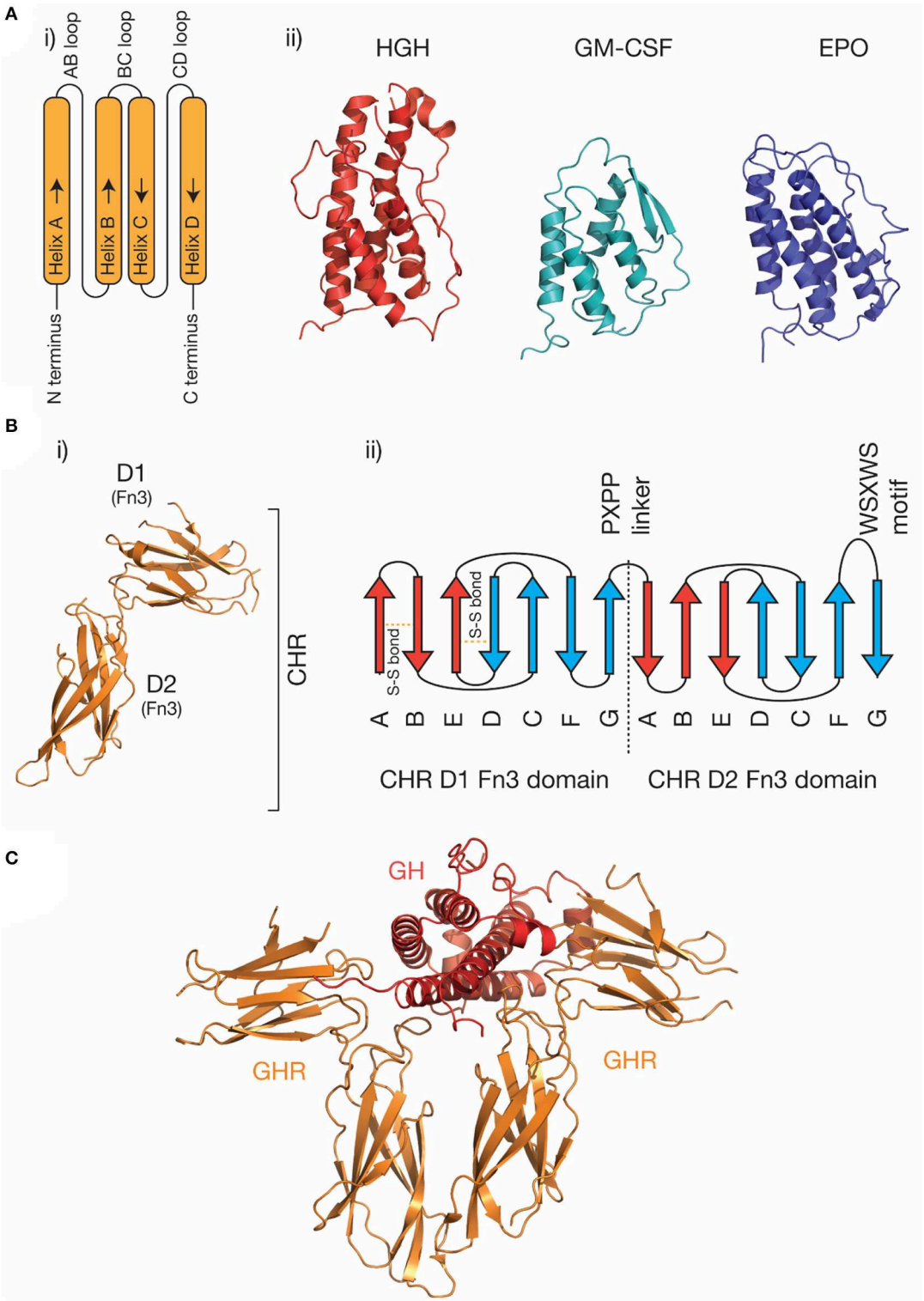

FIGURE 1 | The structure of cytokines and receptors. **(A)** (i) A schematic of the four-α helical bundle topology of hematopoietic cytokines, (ii) cartoon representations of the structures of several representative cytokines; human growth hormone [PDB ID: 1HGU (40)], GM-CSF [PDB ID: 1CSG (41)], and erythropoietin [PDB ID: 1BUY (42)]. **(B)** The structure of the growth hormone receptor [PDB ID: 2AEW (43)]. The two Fn3 domains that make up the CHR are indicated, and a typical topology (30) for the two Fn3 domains in the CHR is shown in (ii). The conserved disulfide bonds in the N-terminal domain, the linker sequence, and the conserved WSXWS motif are indicated. **(C)** The structure of the growth hormone/growth hormone receptor complex [PDB ID: 3HHR (44)].

structure solved was the GH:GHR complex in 1992 (**Figure 1C**), which revealed GH bound to a dimer of GHR (44). The most surprising feature of the structure was the observation that two chemically distinct binding sites on GH bind similar epitopes on GHR. Following the GH:GHR structure, more complex structures followed, such as the tetrameric viral IL-6 (67) complex, the hexameric IL-6 (68) complex, and the dodecameric GM-CSF (69) complex, providing a more thorough understanding of cytokine/receptor engagement from several cytokine families. To date, no high-resolution structures have been solved that include the transmembrane or intracellular regions of cytokine receptors, although low-resolution negative-stain electron microscopy studies have captured the overall organization of these complexes (65, 70, 71).

The use of shared signal transducing receptors by cytokines is common. For example, three cytokines utilize the common β chain (β_c), IL-3, IL-5, and GM-CSF (72), six cytokines utilize the common γ chain (γ_c), IL-2, IL-7, IL-9, IL-13, IL-15, and IL-21 (73), and more than ten cytokines utilize glycoprotein (gp)130, including IL-6, IL-11, leukemia inhibitory factor (LIF), CNTF and oncostatin M (OSM) (74, 75). As structures have now been solved of several representative cytokines from these families, the mechanisms of shared receptor use have begun to be understood. For example, the γ_c receptor has a large binding surface in the CHR, allowing it to bind structurally diverse cytokines (48, 73), in contrast, gp130 has a structurally rigid, chemically diverse binding surface at the CHR, with different gp130-binding cytokines interacting with different but overlapping regions of the surface (76). In shared receptor systems, cytokine-specific receptors with restricted expression, such as IL-6Rα or IL-15Rα, serve to limit the activity of cytokines to specific target cells despite their utilization of similar intracellular signaling pathways.

Intracellular Signal Transduction by Cytokines—The JAK-STAT Pathway

The JAK-STAT pathway is the most well-studied pathway activated in response to cytokines (**Figure 2A**). The major components of the pathway are cytokine, cytokine receptor, kinase (i.e., JAK), signal transducer (i.e., STAT), and negative feedback regulators (i.e., SOCS). JAKs are associated with the cytoplasmic domains of signal-transducing cytokine receptors and consist of four domains, a kinase domain, pseudokinase domain, 4.1 ezrin radixin moesin (FERM) domain, and Src homology 2 (SH2) phosphotyrosine-binding domain. The pseudokinase domain regulates the kinase domain (77), with the term "Janus kinase" referring to the presence of two kinase domains, real and pseudo, named for the two-faced Roman god (21). The FERM/SH2 domains form a single structural unit (78, 79), and are responsible for interacting with the cytokine receptor, through defined motifs on the receptor, termed Box 1 and Box 2 (80). Cytokine binding results in the activation and phosphorylation of the kinases, which then phosphorylate the cytokine receptor at STAT binding sites, serving to recruit STATs. Bound STATs are themselves phosphorylated, resulting in the activation of the STAT dimer, its translocation to the nucleus, and the expression of cytokine responsive genes. Importantly, different kinases are associated with different cytokine receptors—for example, the IFNα/β receptor primarily uses tyrosine kinase 2 (TYK2) (22) and β_c primarily uses JAK2 (81). Furthermore, different receptor-kinase complexes result in activation of different STAT proteins—for example, STAT1/2 for IFNα/βR (22), STAT5 for β_c (81), leading to different gene expression programs in response to signaling.

The SOCS proteins, which are expressed as a consequence of cytokine activation, negatively regulate the pathway (27). The SOCS proteins recruit the E3 ligase, Cullin5, resulting in the degradation of the receptor complex in the proteasome (82, 83). Two SOCS proteins, SOCS1 (84) and SOCS3 (85), also directly inhibit the kinase activity of the JAKs. The protein inhibitor of activated STAT (PIAS) proteins inhibit the activity of STAT through mechanisms that include directly blocking STAT interaction with nuclear DNA (86, 87). Several phosphatases act as negative regulators of signaling, such as the SH2-domain containing phosphatases, SHP1 and SHP2 (88, 89) and protein-tyrosine phosphatase (PTP) 1B (90). The lymphocyte adaptor protein, Lnk, serves as an additional negative regulator of signaling by several cytokines that signal using JAK2 (91).

The exact mechanisms by which cytokine engagement triggers signal transduction remain unclear and are the subject of active investigation. In the classical model of cytokine signaling, dimerization of signal transducing receptors simply brings the associated JAKs close enough in proximity to phosphorylate each other in trans (44, 92) (**Figure 2B**). However, several cytokine receptors, including GHR (43, 93), EPOR (94), and gp130 (95, 96) have been shown to exist as preformed dimers at the cell membrane (**Figure 2C**). Investigations of GHR suggest that cytokine binding results in a rearrangement of the transmembrane α-helices of the receptor, a conformational change that lifts pseudokinase domain mediated inhibition of the JAKs (43, 93). Determining the universality of such a mechanism will require the study of additional cytokine receptors, particularly those that signal through more complex hetero-dimeric or larger signaling complexes.

In addition to the JAK-STAT pathway, cytokines can utilize alternative signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K) pathway (81). The multi-adaptor protein SH2 domain containing tyrosine phosphatase (SHP2) interacts with several cytokine receptors and provides the link between the receptors and the MAPK pathway (97). Signaling through these pathways is generally less well understood than the JAK-STAT pathway.

THE IL-6 FAMILY OF CYTOKINES

The IL-6 family of cytokines is one of the largest cytokine families (**Figure 3**). These cytokines are unified by the near-universal use

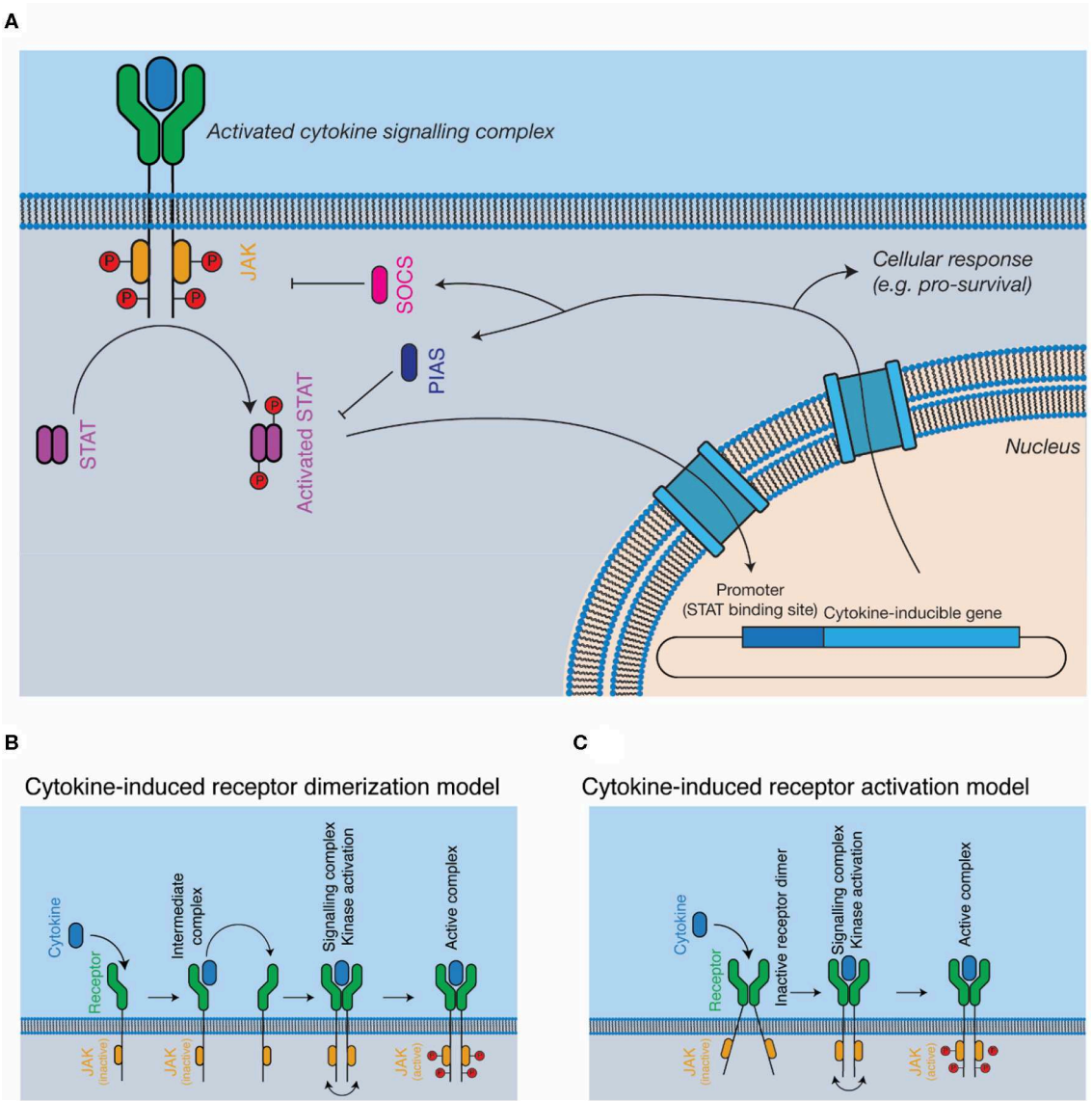

FIGURE 2 | Cytokine signal transduction. **(A)** General schematic of the JAK-STAT pathway. Cytokine binding results in the activation of intracellular kinases (JAKs) that phosphorylate and activate STATs, which subsequently translocate to the nucleus, resulting in altered gene expression, and negative feedback on the pathway through the SOCS proteins. **(B,C)** Models for complex activation. Cytokines are thought to either, **(B)** dimerise receptors on the cell surface, resulting in kinase autophosphorylation and activation or **(C)** bind to pre-dimerised receptors on the cell surface, resulting in receptor activation through conformational alterations of the receptor dimer.

of the shared signal transducing receptor, gp130. The exception is IL-31, which uses the related receptor IL-31Rα, also known as gp130-like receptor (GPL) (102, 103). The distinct biological activity of IL-6 family cytokines is controlled by the restricted expression of the cytokine-specific receptors, such as IL-6Rα and IL-11Rα by a limited subset of cell types (104). Several cytokines can bind IL-6Rα in addition to IL-6, including CNTF (105), the IL-27 subunit IL-27p28 (also known as IL-30) (106), a IL-27p28 fusion with cytokine-like factor (107), and human herpes virus 8 IL-6 (vIL-6) (108), a viral analog of IL-6 with ∼25% sequence identity to mammalian IL-6 (109). Receptor promiscuity is thus a common feature of the IL-6 family.

The Structure of IL-6 and Its Receptors

IL-6 was initially identified under several names in the 1980s (110, 111) as a protein involved in B-cell differentiation (112), a plasmacytoma growth factor (113), and a protein involved in the induction of acute phase proteins in the liver (114). Subsequent cloning of these proteins showed that they were all identical, thus

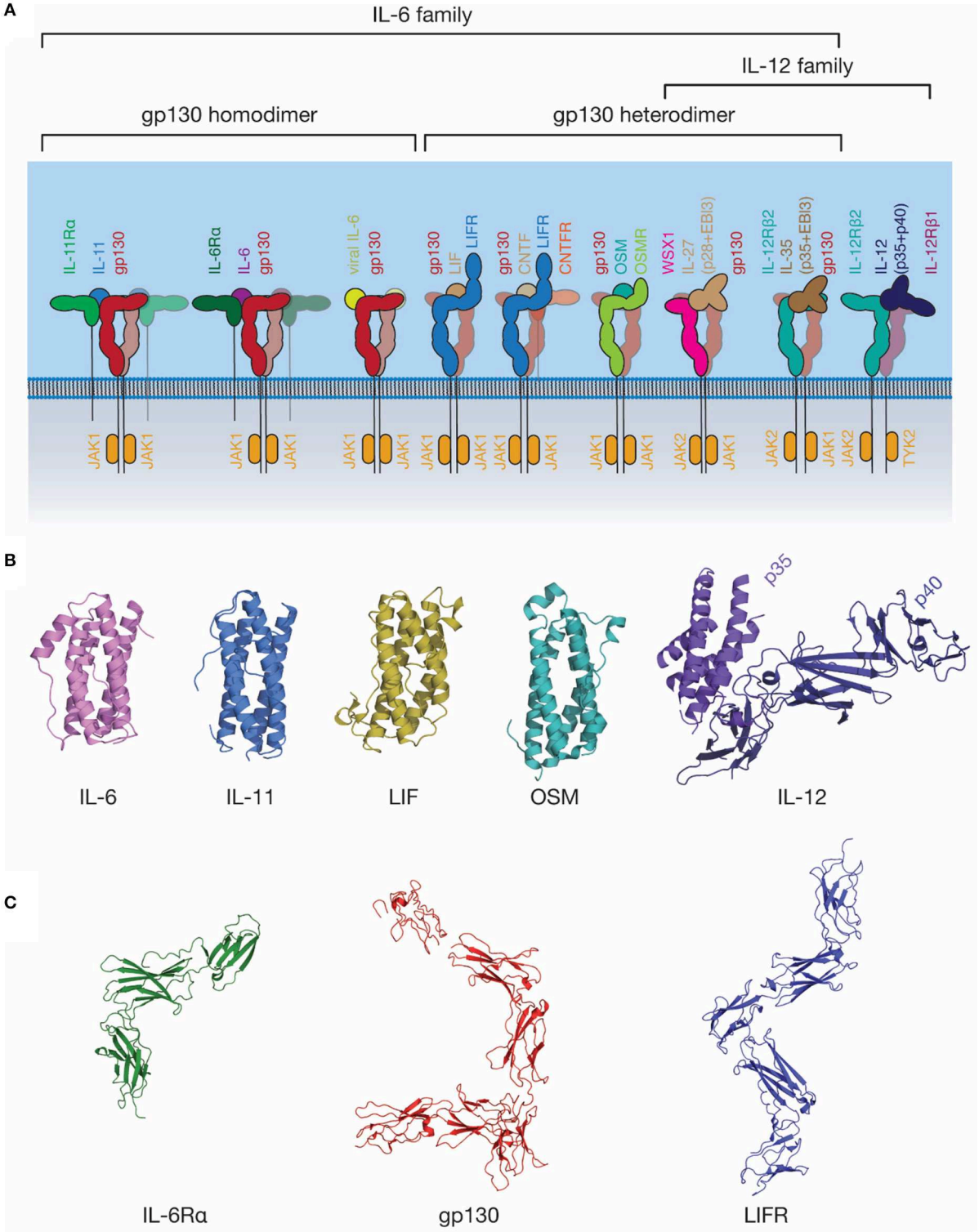

FIGURE 3 | The IL-6 family of cytokines. **(A)** A schematic representation of selected IL-6 and IL-12 family cytokine-receptor complexes, illustrating the diversity in the stoichiometry of signaling complexes employed. Indicative JAK family members utilized by each signal transducing receptor are shown. **(B)** The structures of several IL-6 and IL-12 family cytokines: IL-6 [PDB ID: 1ALU (45)]; IL-11, [PDB ID: 4MHL (98)]; LIF [PDB ID: 1LKI (99)]; OSM [PDB ID: 1EVS (100)]; IL-12 [PDB ID: 1F45 (54)]. **(C)** The structures of extracellular domains of IL-6 family cytokine receptors: IL-6Rα [PDB ID: 1N26 (101)], the common signal transducing receptor, gp130 [PDB ID: 3L5H (58)] and the receptor for LIF and several other IL-6 family cytokines, LIFR [PDB ID: 3E0G (65)].

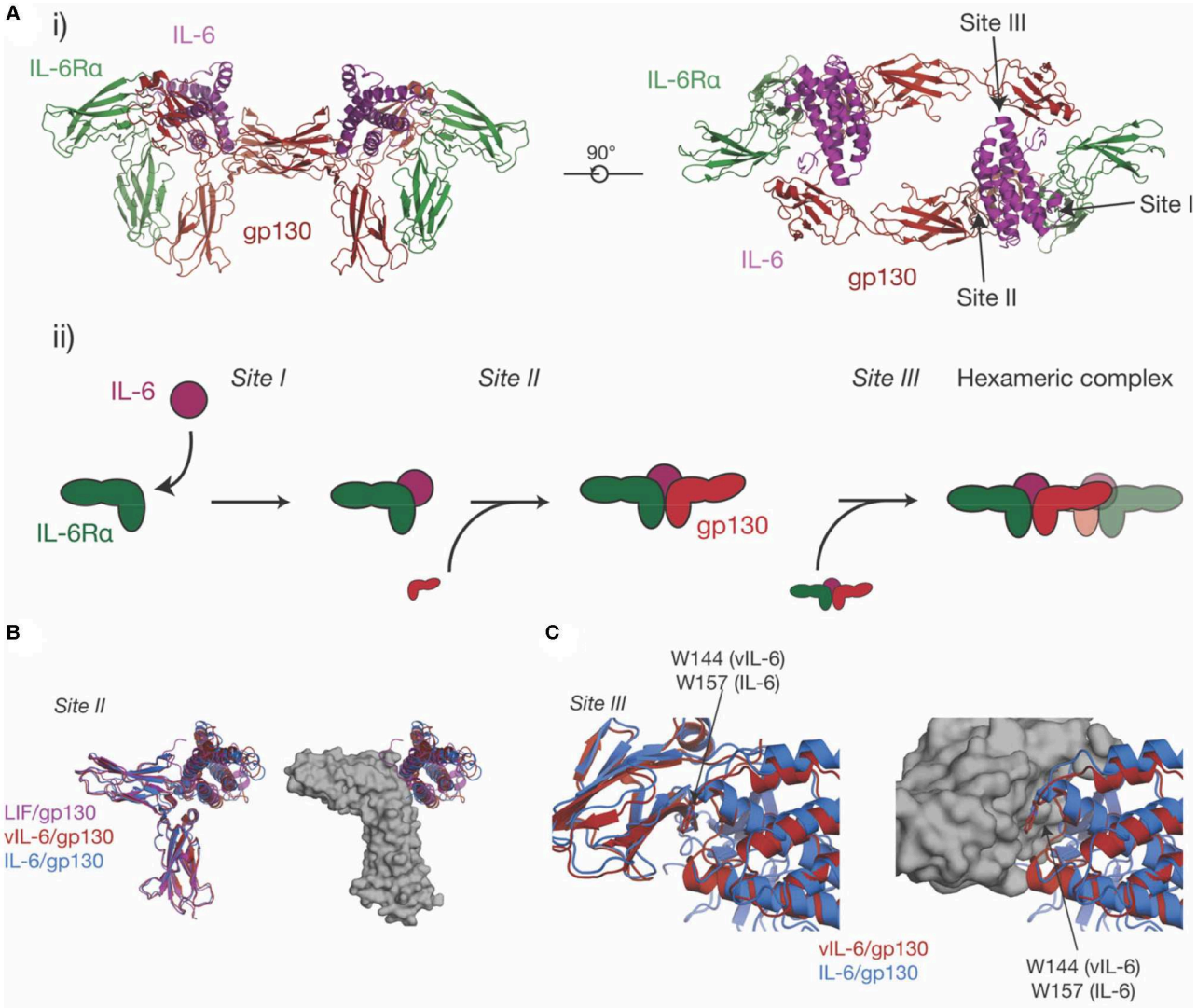

FIGURE 4 | The structure of the IL-6 signaling complex. **(A)** (i) Two views of the structure of the hexameric IL-6/ IL-6Rα/gp130 complex [PDB ID: 1P9M (68)]. The three binding sites on the cytokine (site-I, binding IL-6Rα, site-II, binding gp130, site-III binding gp130), are indicated in the figure. The stepwise assembly of the complex is shown in (ii), with the interactions mediated by each of the binding sites indicated. **(B)** The binding of three IL-6 family cytokines to the CHR of gp130, IL-6, vIL-6 [PDB ID: 1I1R (67)] and LIF [PDB ID: 1PVH (76)]. The three cytokines do not induce significant rearrangements in the CHR of gp130 but adopt a different pose on the CHR and bind different regions in the surface of gp130. **(C)** The binding of IL-6 and vIL-6 to gp130 D1, the interaction that forms the hexameric complex. The two cytokines engage gp130 D1 in an analogous way. The key tryptophan "hot-spot" residue in site-III is indicated.

they were given a common name, IL-6. IL-6 is the most well-characterized member of this family structurally, with crystal structures of IL-6 solved in 1997 (45, 115), the structure of IL-6Rα solved in 2002 (101), and the structure of the IL-6 signaling complex solved in 2003 (68) (**Figures 3B,C**, **4A**). IL-6 is a typical four-α helical bundle cytokine, with the expected up-up-down-down arrangement of α-helices, with an additional, short α-helix in the CD loop (**Figure 3B**). The extracellular region of IL-6Rα consists of three domains (101), an N-terminal Ig-like domain, and two Fn3 domains, which form the IL-6 binding CHR (**Figure 3C**). The N-terminal Ig domain adopts a distorted Ig-like fold, and is dispensable for cytokine binding and biological activity (60, 68), although there is some evidence that it is required for correct trafficking of the receptor (60). IL-6 binds the surface formed by the two Fn3 domains, D2 and D3, comprising the CHR (68). C-terminal of the structured extracellular domains (D1-D3), there is a long linker region (52 residues), predicted to be disordered, that appears to function as a spacer in the signaling complex between the structured extracellular domains and the membrane (116–118).

Gp130 is the common signal transducing molecule for nearly all IL-6 family cytokines, and some cytokines in the closely related IL-12 family. It was first identified in 1989 (119) as the component of the IL-6 signaling complex involved in signal

transduction, and subsequently cloned in 1990 (120). Following this, gp130 was recognized as being a common component of the IL-11 (121), OSM (122), LIF and CNTF (123) signaling complexes. Structures of the CHR domains of gp130 became available in 1998 (124), and the full extracellular region of gp130 in 2010 (58) (**Figure 3C**). The extracellular domains of gp130 are those of a typical "tall" cytokine receptor, consisting of six domains, an N-terminal Ig-like domain, and five Fn3 domains (58). The first three, membrane-distal domains (D1-D3) are involved in cytokine recognition and complex formation, and are sufficient to bind cytokines and form a complex in solution (68, 76). The membrane-distal domains are also directly involved in gp130 activation, with oncogenic mutations that result in cytokine-independent activation of gp130 clustering in D2 (125). These mutations are thought to act by disrupting the D2/D3 interdomain linker, allowing the receptor to adopt an active conformation in the absence of ligand (126).

The three membrane proximal domains of gp130 (D4-D6) are not directly involved in binding the cytokine, but are required for signal transduction, as deletion of any of the domains results in an inactive receptor (127). Electron microscopy shows that the membrane-proximal domains are involved in the correct orientation of the intracellular kinases for signal transduction (65, 70, 71, 128). In addition to the extracellular domains, gp130 contains a large intracellular domain, which is involved in binding molecules required for signal transduction. Structurally, little is known about the intracellular domain of gp130, although NMR studies have shown that the isolated intracellular domain is disordered (65). JAK1, which mediates intracellular signaling, has been shown to bind gp130 at the Box 1 motif in the intracellular domain of gp130 (80). STAT3 (17, 129) and STAT1 (130) bind at C-terminal phosphotyrosine residues in the intracellular domain of gp130 (131). Specifically STAT3 utilizes Tyr767, Tyr814, Tyr905, and Tyr915, while STAT1 utilizes Tyr905 and Tyr915 (132). SHP2 is also recruited by gp130 at the intracellular domain (133), interacting with Tyr759 providing the link between gp130 and the MAPK pathway (134). The same Tyr759 allows for SOCS3 regulation of cytokine signaling (27, 85).

The Structure of the IL-6 Signaling Complex

Prior to the determination of the structure of IL-6 in complex with the cytokine binding domains of its receptors (**Figure 4A**i) (68), there was extensive evidence from analytical ultracentrifugation and electrophoresis that the complex was hexameric, comprising two copies each of IL-6, IL-6Rα, and gp130 (135–137). Concurrently, mutagenic studies identified three binding sites on IL-6 (136), which were later confirmed in the structure of the complex (68). Site-I is responsible for binding IL-6Rα, site-II is responsible for binding the first molecule of gp130, and site-III is responsible for binding the second molecule of gp130, resulting in the formation of the hexameric complex (**Figures 4A**i, ii). Site-I and site-II are positioned on the cytokine in a broadly analogous manner to GH and form a similar trimeric complex, with IL-6 binding the CHRs of IL-6Rα and gp130 (33) (**Figure 4A**i). The distinct cytokine:Ig domain interaction between the cytokine and D1 of gp130 is unique to IL-6 family cytokines (138). This interaction is formed by site-III on the cytokine. The complex is formed by ten interdependent interfaces between IL-6 and the two receptors, and between the receptors, with the earlier binding events creating composite binding surfaces to enable subsequent receptor recruitment. The structure of the IL-6 signaling complex has aided drug design studies (139, 140), showing its value in the design of novel therapeutics.

The site-II/CHR region of gp130 is involved in the binding of all gp130-binding cytokines. Alongside the structure of the IL-6 signaling complex, structures were solved of vIL-6 in complex with gp130 (67) and LIF in complex with gp130 (76). All three cytokines engage the CHR of gp130 *via* the site-II region of the cytokine (**Figure 4B**). The structures showed that vIL-6, IL-6, and LIF engage different but overlapping binding regions in the CHR of gp130, with the three cytokines adopting different poses. A key residue in site-II of gp130, Phe169, forms important interactions with IL-6, vIL-6, and LIF. Surprisingly, the cytokine binding surface of gp130 is relatively rigid, and does not significantly change conformation in response to the binding of different cytokines (76). The CHR of gp130 presents a large, chemically diverse binding surface and the different regions engaged by IL-6, vIL-6, and LIF result in each cytokine/gp130 interaction displaying different thermodynamic properties (76). The size and "thermodynamic plasticity" (76) of the CHR of gp130 is thought to result in its promiscuous binding to multiple cytokines (33, 76).

IL-6 and vIL-6 interact with the Ig-like domain D1 of gp130 through site-III on the cytokine. The interactions between IL-6/gp130 D1 and vIL-6/gp130 D1 are broadly analogous (**Figure 4C**). In both complexes, a conserved tryptophan is the key hydrophobic "hot spot" residue (Trp157 in human IL-6, Trp144 in vIL-6), providing ∼25% of the buried surface area at site-III. Likewise, the N-terminus of gp130 forms a short mainchain-mainchain interaction with the AB loop of the cytokine (67, 68). The site-III interface on gp130 D1 is otherwise relatively chemically and structurally featureless (33), providing a low-affinity binding surface that is reliant on prior interactions with other receptors for stable complex assembly. An interaction similar to the gp130-D1 interaction is formed by LIF with the Ig-D3 and Fn3-D4 of LIFR, although this interaction buries more surface area and forms more polar interactions (59).

No structural data are available for the gp130 binding epitopes of any IL-6 family cytokines other than vIL-6, IL-6, and LIF. Mutagenesis of gp130 shows that IL-11 and IL-6 both require D1 of gp130 for signaling, and bind a similar epitope in the CHR (141). Monoclonal antibodies against gp130 have been developed that antagonize signaling through specific cytokines, including IL-11 and IL-6-specific neutralizing antibodies, suggesting that each cytokine engages gp130 using a structurally different mechanism (142); however, the structural basis of this specificity is currently unknown.

No high-resolution structures are available of the complete extracellular regions of any IL-6 family cytokine complex. All complexes described above comprise heavily truncated forms of the receptors to facilitate crystallization. Electron microscopy

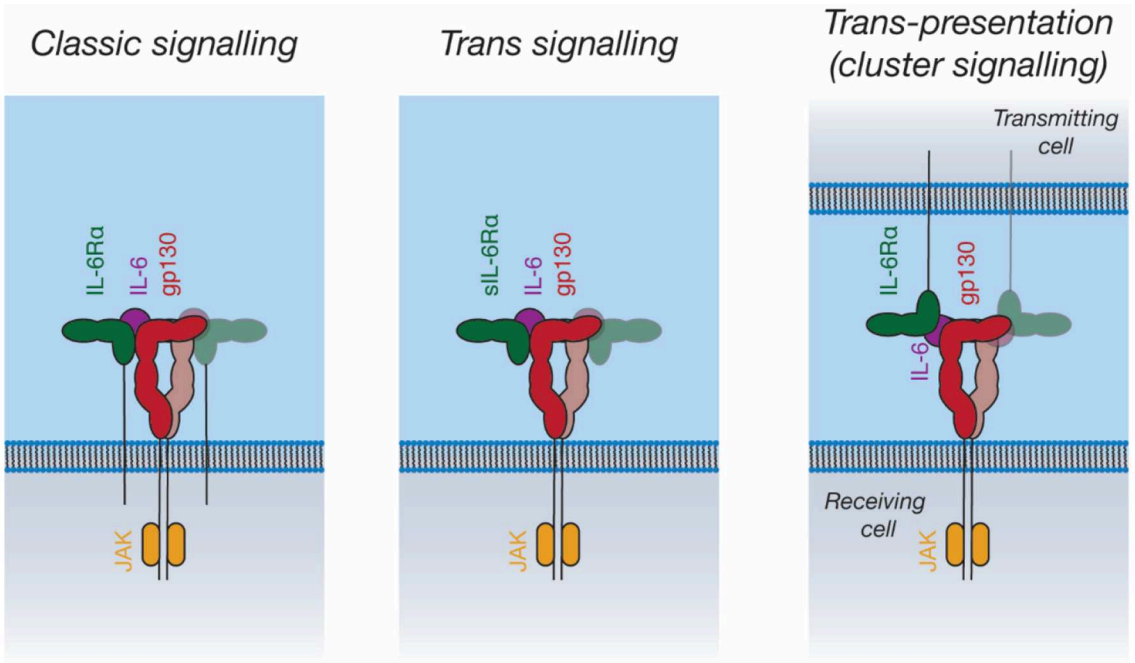

FIGURE 5 | Signal transduction by IL-6. IL-6 can activate intracellular pathways in three ways: "Classic" signaling, in which IL-6 binds to membrane-bound IL-6Rα, and subsequently binds to membrane-bound gp130 on the same cell; "trans-signaling", in which IL-6 binds to soluble IL-6Rα, subsequently binding membrane-bound gp130; and "trans-presentation," in which IL-6 binds membrane-bound IL-6Rα on a "transmitting cell" and subsequently engages gp130 on a neighboring ("receiving") cell, activating intracellular signaling pathways in the receiving cell.

(both cryogenic and negative stain) has been used to study several complexes, including the IL-6 complex (70, 71), the LIF complex (65), and the IL-11 complex (128). The resolution in these studies is insufficient to resolve structural detail of the complex, although they reveal a common "doughnut-shaped" architecture, with the "legs" of the tall cytokine receptors, LIFR and gp130, bent to create a complex with a hole in the middle. The details of any contacts between the membrane proximal domains of the receptors in these complexes remain to be elucidated and will require the determination of high-resolution structures of the complete extracellular regions of the complexes.

Alternative Mechanisms of IL-6 Family Signaling

In addition to "classic" IL-6 signaling through membrane-bound IL-6Rα and gp130, IL-6 can also bind a soluble form of IL-6Rα (sIL-6Rα). The IL-6/sIL-6Rα complex can then engage membrane-bound gp130, allowing the stimulation of cells that do not express IL-6Rα, a process known as *trans*-signaling (119, 143) (**Figure 5**). IL-6 *trans*-signaling is implicated in IL-6 mediated inflammation (143). sIL-6Rα is generated through alternative splicing (144) and through cleavage of the intact receptor by the membrane-bound metalloproteases, ADAM10 and ADAM17, resulting in shedding of the extracellular receptor domains (143). The physiological antagonist of *trans*-signaling is soluble gp130 (sgp130), which can bind to the sIL-6Rα/IL-6 complex extracellularly, thereby neutralizing its cellular activity (145).

IL-11 *trans*-signaling has recently been identified (146). The membrane metalloprotease ADAM10 can cleave IL-11Rα to produce sIL-11Rα, which can engage IL-11 and gp130 in an analogous manner to IL-6/sIL-6Rα (146). To date, no clear biological role has been ascribed to IL-11 *trans*-signaling. In diseases shown to be driven by classic IL-11 signaling, for example gastrointestinal cancers, it has been shown that there is no role for IL-11 *trans*-signaling (147). Likewise, the loss of classic IL-11 signaling is associated with defects in embryo implantation; however, the inhibition of IL-11 *trans*-signaling in mice does not result in infertility (148). Fusion proteins of IL-6 with IL-6Rα and IL-11 with IL-11Rα ("hyper-IL-6 and hyper-IL-11") are used to mimic *trans*-signaling experimentally (149, 150).

Recent studies have proposed a third IL-6 signaling mechanism, *trans*-presentation, whereby IL-6 binds IL-6Rα on a "transmitting cell," which then presents the IL-6/IL-6Rα complex to gp130-expressing cells (**Figure 5**) (151, 152). This was shown to be critical for the differentiation of T_H17 T helper cells, where IL-6/IL-6Rα is presented in *trans* by dendritic cells (151). *Trans*-presentation has also been shown to be possible for IL-11Rα, however a defined biological role for this has not been identified (152). *Trans*-presentation of IL-6 family cytokines has not yet been characterized structurally; such a signaling mode would require large rearrangements of the IL-6 signaling complex components. Other cytokines such as IL-2

(153) and IL-15 (154) can utilize similar *trans*-presentation mechanisms, where dendritic cells present the cytokine in *trans* to antigen-specific T-cells (48, 155).

Related Cytokine Families

The IL-12 Family of Cytokines

The IL-12 family of cytokines is closely related structurally to the IL-6 family of cytokines, indeed, it has been suggested that a clear distinction between the two families is almost impossible to define (156). In contrast to the majority of the IL-6 family, all IL-12 family cytokines consist of two subunits, a smaller four-α helical subunit, and a larger all-β protein cytokine receptor subunit, which is analogous to the α-receptors for IL-6 and IL-11. For example, IL-12 consists of two subunits, p35, analogous to a four-α helical bundle cytokine, and p40, which resembles a class I cytokine receptor (**Figure 3B**) (54).

IL-27 and IL-35 are two IL-12 family cytokines that utilize gp130 as a signal transducing molecule and, thus, are also grouped as members of the IL-6 family (**Figure 3A**) (157, 158). IL-27 consists of a complex of IL-27p28 and Epstein–Barr virus-induced gene 3 (EBI3) that signals through a heterodimer of WSX1 and gp130 (**Figure 3A**) (157). In addition to this complex, IL-27p28 may utilize IL-6Rα as the cytokine-receptor subunit to signal through a gp130 dimer (106). IL-27p28 was also shown to antagonize IL-6 and IL-27 signaling through gp130, but not OSM signaling, suggesting that IL-27p28 may compete with cytokines that bind D1 of gp130 (159). IL-35 can signal using a heterodimer of IL-12Rβ2 and gp130, or homodimers of either IL-12Rβ2 or gp130; however, the molecular mechanisms underpinning this promiscuity are currently unclear (158). Broadly, these findings suggest an evolutionary relationship between the IL-6 and IL-12 families of cytokines and underscore the promiscuity of cytokine receptors in the IL-6/IL-12 superfamily.

Domeless

A distant homolog of gp130 has been identified in *Drosophila melanogaster*, the receptor *domeless* (*dome*) (160), which is the likely evolutionary ancestor to all IL-6 family cytokine receptors (161). *Dome* shares a similar domain structure to gp130 and LIFR, and has a putative CHR, albeit with low sequence identity to the CHR of gp130. A putative ligand for *Dome*, *Unpaired-3* (*Upd3*) (162) has also been identified, alongside JAK kinases (*Hopscotch*) and STAT transcription factors (*Marelle*) (163). The *Dome-Hopscotch* pathway has been shown to have several roles in *Drosophila* physiology, including in responding to bacterial infection (164), in oogenesis (164), in hemocyte proliferation (165), and in tissue development (166, 167), showing that cytokine pleiotropy is a common feature in metazoans. Neither *dome* or *Upd3* have been studied structurally, although recombinant *Upd3* has been produced, and has been shown by circular dichroism spectroscopy to have a predominately α-helical secondary structure (168). Zebrafish possess a mammalian-like cohort of cytokines, with relatives of all extant mammalian cytokine families present, suggesting that an increase in diversity of cytokines and receptors occurred with the evolution of the adaptive immune system in vertebrates (169, 170).

BIOLOGICAL ROLES OF IL-11

IL-11 was first identified in 1990, following the discovery of a protein factor that stimulated a murine plasmacytoma cell line previously thought to be IL-6 dependent (171). The following year, IL-11 was also identified as a factor secreted from a bone marrow derived cell line culture, which inhibited adipogenesis in preadipocytes (172, 173), thus the pleiotropic nature of IL-11 signaling was appreciated early. While there was a flurry of activity surrounding IL-11 in the 1990s, there has been less research activity since. However, in the last decade there has been a renewed interest in IL-11 following its emerging role in numerous diseases.

Structure of IL-11 and Its Receptors

In contrast to IL-6, LIF and other IL-6 family cytokines, little was previously known about the structure of IL-11 or IL-11Rα. We reported the first crystal structure of IL-11 in 2014 (98) and have recently reported a higher-resolution structure of the cytokine (**Figure 6A**) (174). Our structures show that IL-11 is ∼5 Å longer than IL-6, suggesting differences in binding mode and geometry within the signaling complex. Likewise, the IL-11Rα binding site (site-I) and the first gp130 binding site (site-II) of IL-11, previously identified through mutagenesis (175, 176), are different in chemical character to IL-6, with site-I more hydrophobic (**Figure 6B**). Our recent structure of IL-11Rα (**Figure 6C**) (174) revealed that the cytokine binding site of the receptor is more hydrophobic in character than IL-6Rα, consistent with the corresponding site of IL-11 and suggesting distinct mechanisms of cytokine engagement.

No high-resolution structural data for the IL-11 signaling complex are currently available in the literature, although sequence analysis (121, 177) and our structural data (174) show that IL-11Rα and IL-6Rα are structurally similar. The IL-11 signaling complex, like the IL-6 signaling complex, is thought to be hexameric, as shown by immunoprecipitation and electrophoresis (178). Contemporaneous mutagenic studies (175, 176, 179) also identified site-I, II, and III on IL-11 (**Figure 6B**), suggesting that IL-6 and IL-11 form an active signaling complex using a broadly similar mechanism. A low-resolution (∼30 Å) cryoEM density map of the IL-11 signaling complex extracellular domains (128) (**Figure 6D**) shows that the overall arrangement of the complex is broadly similar to the IL-6 signaling complex (**Figure 6D**ii), although the details of complex formation were not clear at this resolution. We have recently solved structures of the IL-11 signaling complex that provide high resolution detail of the assembled complex (unpublished).

IL-11 in Hematopoiesis

Early studies of IL-11 revealed that it was a potent hematopoietic factor, acting synergistically in culture with other cytokines, such as IL-3 (180, 181) and IL-4 (182). In particular, IL-11 was found to have a role in megakaryocytopoiesis, causing the maturation of megakaryocytes, large cells which form platelets (181). In mice, IL-11 alone is a potent hematopoietic stimulator

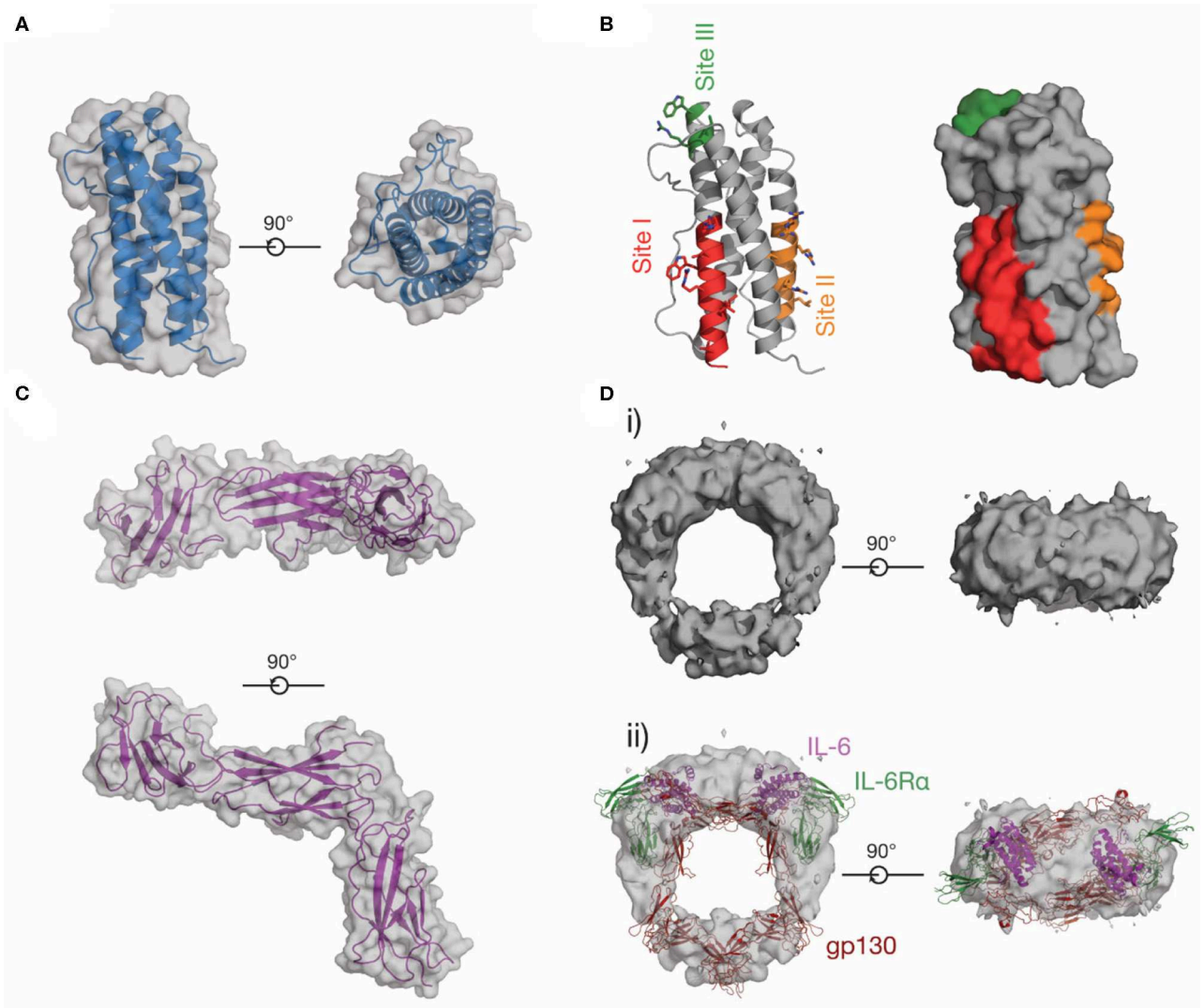

FIGURE 6 | The structure of IL-11. **(A)** Two views of the structure of IL-11 [PDB ID: 6O4O (174)]. **(B)** Receptor-binding sites on IL-11, which have been identified through mutagenic studies on human and mouse IL-11. **(C)** Two views of the structure of the extracellular domains of IL-11Rα [PDB ID: 6O4P (174)]. **(D)** A low-resolution EM map of the extracellular IL-11 signaling complex [EMD-1223 (128)] (i), additionally shown overlaid with a model of the extracellular IL-6 signaling complex, (ii) [generated from PDB IDs: 3L5H (58), 1P9M (68)].

following radiation therapy and chemotherapy, and markedly increases platelet counts (183). Recombinant IL-11 is approved by the FDA to treat thrombocytopenia following radiation treatment in humans (184), and is commonly prescribed to breast cancer patients. In addition to its well-characterized role in megakaryocytopoiesis, IL-11 has other roles in hematopoiesis (185), for example, in lymphopoiesis (186), in erythropoiesis (187), and in myelopoiesis (188).

IL-11 in Bone Development

IL-11 signaling has been shown to promote osteoblast differentiation, and thus bone formation, with IL-11Rα knockout mice showing abnormal craniofacial features (189–191). In humans, mutations in the genes for IL-11 and IL-11Rα are associated with a reduction in height (192, 193), suggesting that IL-11 signaling has a role in regulating growth. Likewise, a genetic variant in the gene for IL-11, resulting in a substitution mutation (R112H), is associated with osteoarthritis and a reduction in height (192, 194). Biochemical characterization of the mutant cytokine has shown that it does not alter the biological activity of IL-11, but compromises the stability of the protein (195).

Over the past decade, a number of studies have identified mutations in the gene for IL-11Rα, which result in a genetic disease associated with craniosynostosis (196–198). Craniosynostosis is a condition in which bone plates in the skull fuse too early, resulting in facial abnormalities and an abnormally shaped skull. The disease is rare, and has been found

in families with diverse geographic origins (196). Generally, the disease occurs as a result of point substitution mutations in the extracellular domains of IL-11Rα (196, 199), and many of these mutations are situated in regions distant from the putative cytokine or receptor binding sites. Several of the mutations have been shown to impair correct processing and surface expression of the receptor (199). Molecular dynamics simulations using our IL-11Rα structure indicate that some mutations destabilize the receptor and may have indirect effects on the cytokine binding region (174).

IL-11 in The Lung

IL-11 is highly expressed as a consequence of viral induced asthma (200), and overexpression of IL-11 in the airways of mice results in remodeling of the airways, inflammation and asthma-like symptoms (201). Subsequent studies have shown that IL-11 signaling is critical for a T_H2-mediated inflammatory response in the lung (202), and that inhibition of IL-11 signaling in the lung alleviates inflammation, implying that IL-11 signaling is a therapeutic target in asthma (203). Similarly, IL-11 has been shown to drive lung inflammation in a murine model of *Mycobacterium tuberculosis* infection (204).

IL-11 in Reproduction

Female knock-out mice lacking the gene for IL-11Rα are infertile, and cannot undergo the uterine transformations required for embryo survival (205). Likewise, IL-11 and IL-11Rα have been localized to reproductive tissues during early pregnancy in primates, suggesting a role in placentation and decidualization (206). Related to this, inhibition of IL-11 signaling impairs decidualization and prevents pregnancy in mice, suggesting that therapeutic inhibition of IL-11 may be a potent contraceptive (207). Defects in the production of IL-11 have also been associated with anembryonic pregnancy, a cause of miscarriage (208). IL-11 signaling inhibits and regulates invasion of extravillous trophoblasts, cells which are key in placentation for the formation of blood vessels (209–211). Thus, elevated IL-11 is associated with preeclampsia, a disease where placentation is impaired, resulting in hypertension (211). Together, these studies suggest that IL-11 has key roles in driving the tissue transformations that occur as a result of pregnancy.

IL-11 in Fibrosis

IL-11 has been implicated in fibrosis of the heart (212), liver (213), and lung (214, 215). Fibrosis is the generation of excess connective tissue, and is a hallmark of several diseases, including late-stage cardiovascular disease, and liver diseases such as non-alcoholic liver disease. In the heart, IL-11 has recently been identified as a key fibrotic factor, acting downstream of the main fibrotic factor TGFβ1, driving fibrotic protein synthesis in an autocrine manner (212). IL-11 has a similar role in driving inflammation and fibrosis of the liver (213). Interestingly, in both cases, the effect has been shown to be driven by non-canonical signaling via the MAPK/ERK pathway, rather than *via* the JAK-STAT pathway. Surprisingly, canonical IL-11 signaling via STAT3 has previously been ascribed a cardioprotective role, inhibiting cardiovascular fibrosis and preventing cardiovascular remodeling following myocardial infarction (216). These contradictory observations may be a consequence of the source of IL-11 used in either study, as it was shown that human IL-11, previously used to show that IL-11 is cardioprotective, does not activate mouse cardiac fibroblasts, while murine IL-11 strongly activates murine cardiac fibroblasts (212). Alternatively, it may suggest different roles for IL-11 in response to different cardiovascular stresses. More broadly, this may reflect an inadequate understanding of the species-specific effects of IL-11, or differences in signaling in humans as compared to mice.

IL-11 in Cancer

IL-11 signaling drives several cancer hallmarks (217, 218) including cell survival, metastasis, and invasion (219–221). IL-11 levels are significantly higher in a murine model of gastric cancer (222), and IL-11 is the major factor that drives STAT3 activation and corresponding inflammation in murine gastric and colon cancer models, as well as human cell line xenograph models of these cancers (221). A role for IL-11 signaling in breast cancer has been less well-described, but elevated levels of IL-11 and IL-11Rα are associated with poor patient outcomes (223, 224) and both IL-11 and IL-6 are associated with breast cancer metastasis into bone (225). IL-11 is also associated with endometrial cancer, and is associated with increasing tumor grade (226). Elevated levels of IL-11 are found in several other types of cancer, including pancreatic cancer (227), skin cancer (228), and bone cancer (229), although a precise role for IL-11 signaling in many of these cancers remains to be defined.

THERAPEUTIC TARGETING OF IL-6 FAMILY CYTOKINE SIGNALING

Given the role of cytokine signaling in numerous pathological conditions there is broad interest in the development of therapeutic agents that block their activity. Generally, inhibition can occur at several points in the cytokine signaling pathway—either by preventing the protein-protein interactions on the cell surface, or by targeting components of the signal transduction machinery within the cell. Conversely, recombinant cytokines can also be used to therapeutically boost cytokine signaling. Here we provide an overview of several approaches to therapeutically modulate cytokine signaling that are in development, as well as those currently used in the clinic. We focus our discussion on how advances in these areas may inform the design of IL-11 signaling inhibitors suitable for clinical use.

Small Molecules

Inhibitors of Intracellular Signal Transducing Proteins

JAK inhibitors are widely used, orally bioavailable, small molecules for the treatment of blood cancers and inflammatory diseases (230) (**Figure 7**). Six JAK inhibitors are used clinically, with several in development. For example, the JAK1/2 selective inhibitor ruxolitinib (231) is used to treat a group of rare blood cancers associated with an activating mutation in JAK2. Similarly, tofacitinib (non-selective) and baricitinib (selective for JAK1/2) are JAK inhibitors used to treat the inflammatory

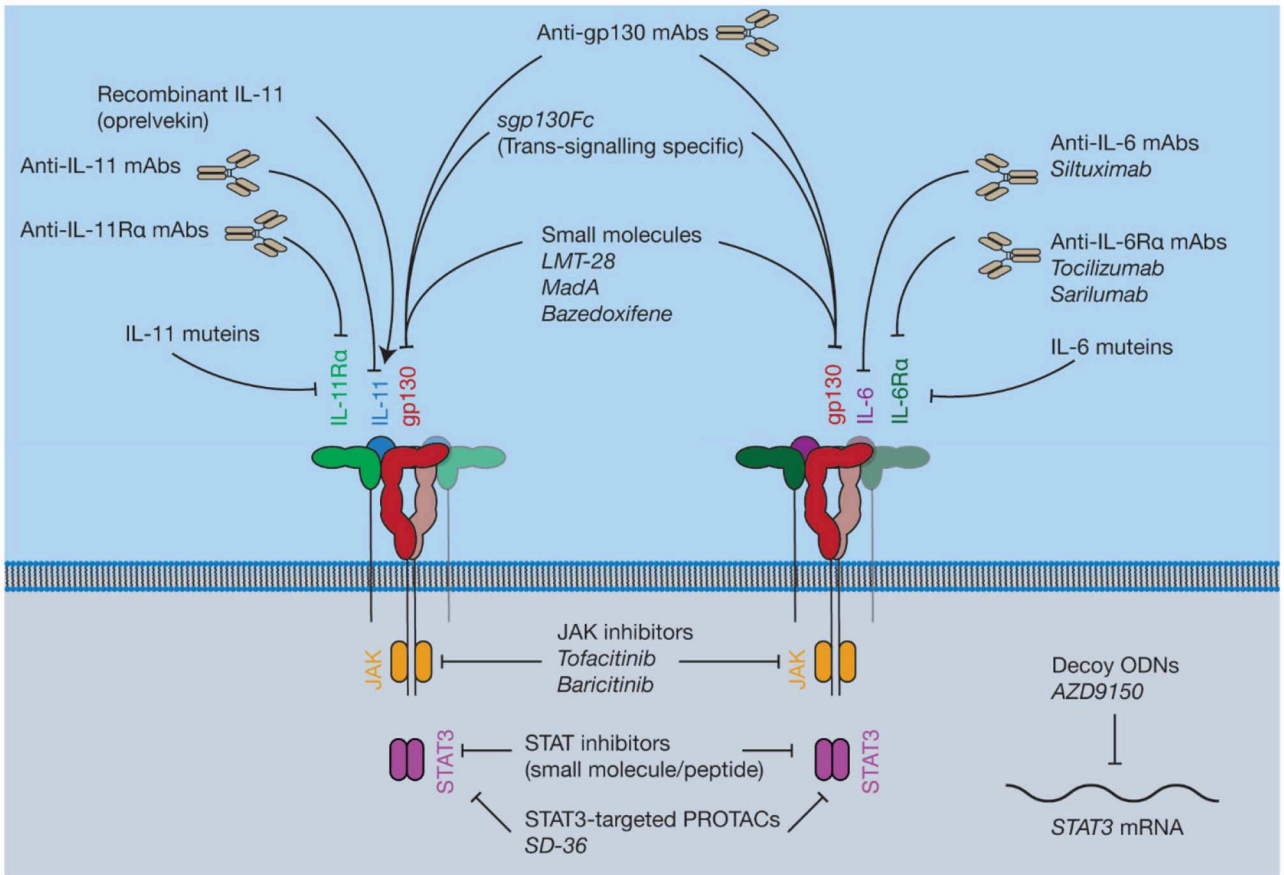

FIGURE 7 | Pharmacological approaches to target IL-6 and IL-11 signaling. Current inhibitors of IL-6 and IL-11 signaling include protein antagonists such as cytokine mutants and antibodies, small molecule protein-protein interaction (PPI) inhibitors targeting gp130, recombinant IL-11, small molecule inhibitors of proteins in the intracellular JAK-STAT pathway, and decoy oligodeoxynucleotides (ODNs) targeting the *STAT3* mRNA.

disease rheumatoid arthritis (232, 233). JAK inhibitors are now undergoing clinical trials for a broader array of inflammatory diseases (234). Challenges with developing JAK inhibitors are largely a consequence of the inherently non-specific nature of the drugs. Moreover, JAK inhibition may be associated with severe side effects, including opportunistic viral infections, likely a consequence of inhibition of interferon-mediated protective antiviral signaling (235). Similarly, due to the central roles of cytokine driven JAK activation in hematopoiesis, JAK inhibitors have been noted to cause mild anemia and neutropenia (236, 237). Despite this, JAK inhibitors are widely used, and efforts to develop novel JAK inhibitors, particularly inhibitors that are selective for a specific kinase, are ongoing.

Inhibitors of STAT activity are in various stages of development (238). Phase I and II trials have been conducted on several drug candidates targeting STAT3, although the results are pending (239, 240). These inhibitors are generally peptides or small molecules designed to prevent STAT dimerization (241, 242), or decoy oligodeoxynucleotides (ODNs) designed to target expression of the STAT gene directly (243). Recently, a small-molecule proteolysis targeting chimera (PROTAC), SD-36 (244), which selectively targets STAT3 over other STAT family members, has been described. Direct inhibition of activated STATs is at a less advanced stage compared to kinase inhibitors, or drugs targeting the cytokine/receptor interaction directly, with current inhibitors having low potency and poor pharmacokinetic properties (245). For example, curcumin, an extract of the turmeric plant, *Curcuma longa*, has been used in traditional medicine for centuries for its anti-inflammatory properties (246). Mass spectrometric and computational docking studies have shown that curcumin directly interacts with STAT3 to inhibit phospho-STAT3 dimerization (247). Several *in vitro* studies demonstrate that curcumin is an inhibitor of STAT3 signaling (247, 248). However, the use of curcumin as a drug candidate or treatment is controversial (246, 249). Generally, direct targeting of STATs may not have clear benefits over existing therapeutic strategies, such as JAK inhibitors, which may hinder clinical uptake of STAT inhibitors.

Inhibitors of Signaling Through gp130

Several small molecules have been described that are believed to bind to gp130 and inhibit the protein-protein interactions

(PPIs) that result in complex formation (**Figure 7**). Despite the challenges of targeting PPIs, as they present large flat binding surfaces (250), small molecule modulation of PPIs is potentially invaluable therapeutically. Small molecule inhibitors could be more specific for the inhibition of signaling through individual cytokines compared to JAK inhibitors, which modulate the signaling of numerous cytokines. Moreover, PPI-inhibitors would likely be cheaper, orally bioavailable, and have a shorter half-life compared to biologic therapies, which is beneficial in the event of serious adverse events (251).

Madindoline A (MadA), a natural product isolated from *Streptomyces nitrosporeus* culture, is a small molecule shown to specifically inhibit the activity of IL-6 and IL-11 *in vitro* (252). MadA has subsequently been shown to inhibit the action of IL-6/IL-11, but not LIF, in bone resorption and macrophage differentiation (253). Additional studies have shown that MadA binds specifically to gp130, with a low affinity (254). Chemical synthesis of MadA is difficult (255) and it is produced in low yields by bacterial fermentation, limiting its potential as a drug candidate for large scale production.

The small molecule gp130 inhibitor SC144 was identified serendipitously from efforts to design a human immunodeficiency virus (HIV) integrase inhibitor, which would be a potential anti-HIV drug (256, 257). Several candidate HIV integrase inhibitors were highly cytotoxic (258). A library was built from these cytotoxic molecules (256) and further screening and lead optimization resulted in the synthesis of SC144 (257), which was effective against a variety of cancer models (259). Subsequently, it was shown that SC144 binds gp130 and inhibits the activity of IL-6 and LIF, likely through binding the CHR of gp130, resulting in suppression of cancer growth in human ovarian cancer xenographs (260). Following this initial description of its activity, SC144 has been used by various groups as an experimental tool to block IL-6 signaling through gp130 [see for example (261–263)].

Another small molecule inhibitor that has been shown to bind to gp130, LMT-28, was identified by screening a library of ∼1,000 compounds (264). Computational docking suggested that LMT-28 binds to D1 of gp130, and the putative binding region in D1 of gp130 was supported using site-directed mutagenesis (265). Likewise, SPR showed that LMT-28 specifically bound gp130, with a dissociation constant (K_D) of 7.4 μM, and LMT-28 was able to out-compete IL-6/IL-6Rα for gp130 binding (264). LMT-28 has been shown to specifically inhibit IL-6/IL-11 driven cell proliferation, and block IL-6-driven inflammation *in vivo* (264). In contrast, LMT-28 does not inhibit OSM/LIF activity, consistent with a binding site in D1 of gp130 (264).

Bazedoxifene is an FDA-approved selective estrogen receptor modulator used clinically in combination with other drugs to treat osteoporosis in elderly women (266). It was recently shown that bazedoxifene inhibited gp130 signaling, following an *in silico* screen on the IL-6/gp130 site-III interface (139). Bazedoxifene has been shown to suppress STAT3 activation through IL-6, inhibit tumor growth in a murine model of rhabdomyosarcoma, a soft-tissue sarcoma (267), and inhibit the proliferation of IL-6 dependent cell lines (268). Bazedoxifene has also been shown to block STAT3 activation by IL-11 in human cancer cell lines, and reduce the tumor burden in murine models of gastric cancer (140). Bazedoxifene was also shown to inhibit IL-6 signaling in triple negative breast cancer cell lines (269), and in murine models of the inflammatory cardiovascular disease abdominal aortic aneurysm (270). Recently, more efficacious analogs of bazedoxifene have been synthesized (271). Given that bazedoxifene is already used clinically, and thus has an established safety profile, it represents a potential small molecule inhibitor of both IL-11 and IL-6 signaling that could be used therapeutically.

Biologics

Recombinant Cytokines

Generally, with some exceptions, recombinant cytokines have not seen wide use therapeutically. Although rare, long-term treatment with recombinant cytokines can result in the generation of endogenous antibodies against the cytokine (272). More generally, the pleiotropic nature of many cytokines may result in unpredictable and intolerable inflammation-associated side-effects, which could limit the use of recombinant cytokines in the clinic (273, 274).

Recombinant human IL-11 (oprelvekin) was FDA-approved in 1998 (184, 275, 276) for the treatment of thrombocytopenia (low platelet levels) in myelosuppressive chemotherapy, as a substitute for platelet transfusions. Oprelvekin has also undergone a clinical trial for use thrombocytopenia in myelodysplastic syndrome, in which the bone marrow fails to properly mature blood cells (277). Oprelvekin is, however, not widely used, both for reasons of cost (278) and due to toxicity associated with mild anemia, periostitis, edema and in some cases neuropathy (279, 280). This toxicity can be managed by limiting the dose of oprelvekin (281). IL-11 also has anti-inflammatory properties, and oprelvekin has also undergone small clinical trials in inflammatory bowel disease (282) and rheumatoid arthritis (283). Both trials were inconclusive, and no further trials for either of these indications have been published.

Monoclonal Antibodies

Numerous monoclonal antibodies (mAbs) are used clinically to target IL-6 signaling (284), for example, the anti-IL-6Rα mAbs tocilizumab (285) and sarilumab (286), and the anti-IL-6 mAb siltuximab (287) are used to treat several diseases including rheumatoid arthritis and kidney cancer (**Figure 7**). Antibodies targeting IL-6 signaling are generally well-tolerated but have been noted to result in adverse events. For example, long-term clinical trials have noted that tocilizumab treatment can result in opportunistic infection, neutropenia and gastrointestinal disorders (288, 289), likewise infection, fatigue and neutropenia have been noted as potential adverse effects of siltuximab (290). The anti-IL-6 mAb olokizumab is currently undergoing a phase III clinical trial for rheumatoid arthritis (ClinicalTrials.gov identifier NCT02760368). Structures show that the olokizumab Fab blocks site-III of IL-6, preventing formation of the IL-6 hexameric complex (291). Structures have also been solved of two anti-IL-6 Fabs, which bind site-I, mimicking the IL-6/IL-6Rα interaction (292). No structures are available of the

FDA-approved anti-IL-6 signaling antibodies in complex with their antigen.

Viral infections, including influenza (293), and severe acute respiratory syndrome (SARS) (294, 295), caused by SARS-coronavirus (CoV), can induce cytokine release syndrome (often referred to as "cytokine storm"), a severe immune reaction frequently associated with elevated serum IL-6 (296, 297). Severe coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2 (298), is associated with elevated serum IL-6 and cytokine release syndrome (299–301). Thus, IL-6 signaling inhibition may be a strategy for managing severe and critical COVID-19 (302). Accordingly, tocilizumab is currently undergoing several expedited clinical trials in severe and critical COVID-19 patients (for example, ChiCTR ID: ChiCTR2000029765, ChiCTR2000030894; ClinicalTrials.gov ID: NCT04315480, NCT04317092, NCT04372186, NCT04320615) (303). Tocilizumab appears to reduce mortality in severe and critical COVID-19 patients (300, 304–307), however in some cases poor outcomes have been noted (308).

Antibodies against IL-11 (214, 309) and IL-11Rα (213, 310, 311) that inhibit IL-11 signaling have been described and patented, although none are clinically available. The mechanisms of action of these antibodies have not been described in the literature.

Antibodies against gp130 have been described (142) that specifically antagonize signaling through a specific cytokine or cytokines, although they are not used in the clinic. The structural basis of this specificity is currently unknown, although epitope mapping studies have been conducted on the antibodies (142, 312), which show that the IL-11-specific mAb, B-P4, binds the membrane proximal region (D4-D6) of gp130 and not at the CHR. The OSM/LIF-specific mAb (B-K5), CNTF-specific mAb (B-P8) and broadly neutralizing mAb (B-R3) bind at the CHR of gp130, presumably sterically interfering with cytokine binding (142, 312).

Soluble gp130

Many of the harmful, pro-inflammatory effects of IL-6 signaling are believed to be caused by *trans* IL-6 signaling (143). Soluble gp130 (sgp130) is an antagonist of trans IL-6 signaling (145). Sgp130 fused to an IgG Fc fragment (sgp130Fc, olamkicept) is currently under development as an IL-6 *trans*-signaling specific inhibitor (313). The effect of sgp130Fc treatment has been studied in animal models of a number of inflammatory diseases including several cancers (314, 315), arthritis (316, 317), inflammatory bowel disease (318, 319), and pancreatitis-associated lung inflammation (320). The side effects of existing treatments targeting IL-6 signaling in humans are believed to result from a blockade of classic signaling, resulting in an increased susceptibility to infections, due to the key role of IL-6 signaling in responding to infection (313, 321). In animal models, blockade of IL-6 *trans*-signaling does not alter the IL-6 dependent response to infection (321). Sgp130Fc is currently undergoing phase II clinical trials for colitis (313) (ClinicalTrials.gov ID: NCT03235752; DRKS-ID: DRKS00010101). An anti-*trans*-signaling nanobody has also been developed (322) which specifically recognizes an epitope formed between IL-6 and IL-6Rα, although the structural mechanism behind inhibition has not been described. IL-11 *trans*-signaling has not been ascribed the same biological significance as IL-6 *trans*-signaling, regardless, sgp130Fc is used as a tool to study IL-11 *trans*-signaling (146), and may be a useful therapy in the case that IL-11 *trans*-signaling is found to be pathologically important.

Cytokine Mutants and Designer Proteins

In the past decades, systematic mutagenesis or phage display was used to generate antagonistic variants of IL-6, IL-11, and LIF by altering affinity to IL-6Rα, IL-11Rα, LIFR, or gp130 (203, 323, 324). These antagonists generally function by selectively increasing affinity to one cytokine receptor, and decreasing affinity to a second cytokine receptor, allowing the non-signaling competent mutant to compete with endogenous cytokine for its receptor. For example, a LIF mutein (324) was developed using phage display to increase the affinity for LIFR, while incorporating mutations that reduced the affinity for gp130. This enables the LIF mutein to compete with endogenous LIF for LIFR binding, while the LIF mutein has reduced capacity to form signaling complex with gp130, resulting in inhibition of signaling by LIF. A similar approach was used to design an IL-11 mutein (203). The mutein incorporates two sets of mutations, a mutation in site-III to reduce binding to gp130, and mutations in the AB loop intended to increase affinity to IL-11Rα allowing the IL-11 mutein to compete with endogenous IL-11 for IL-11Rα, and reduce signaling through IL-11.

Recently, a novel CNTF signaling agonist, IC7, was designed (325) by substituting site-III on IL-6 with site-III on CNTF (which binds LIFR), resulting in a cytokine that signals through a gp130/LIFR heterodimer, while being dependent on IL-6Rα, a signaling mode which is not used by any known IL-6 family cytokine (325). Recombinant CNTF has undergone clinical trials previously to treat type-2 diabetes (326), however the trials were halted due the potential immunogenicity of recombinant CNTF. IC7 provides a therapeutic benefit in animal models of diet-induced obesity, and was not observed to have any severe inflammatory or immunogenic side-effects, suggesting that IC7 holds promise as a novel cytokine treatment for diabetes (325).

An additional approach to develop cytokine signaling modulators is the use of computationally *de novo* designed proteins. A notable recent example of the use of protein design is in the development of IL-2 signaling modulators (327). *De novo* designed proteins, which have low sequence identity to endogenous cytokines, can reduce the risk of immunogenicity when using recombinant cytokines or cytokine mutants as drugs. The use of *de novo* protein design may allow the development of IL-11 agonists or antagonists with low immunogenicity that are more potent than existing therapies.

CONCLUDING STATEMENTS

As new roles for cytokines in disease are discovered, the development of therapeutics to inhibit their action invariably follows. Our rapidly increasing understanding of the importance

of IL-11 signaling in disease underscores its potential as a therapeutic target. However, the development and appropriate characterization of inhibitors of IL-11 signaling has not matured at the same pace. Detailed biophysical and structural information obtained in parallel with pre-clinical testing can greatly facilitate design, specificity, and potency of new cytokine inhibitors, ensuring that the best therapeutics are entered into clinical trials. Thus, improved structural and molecular understanding of the IL-11 signaling complex and current generation inhibitors will be of great benefit for therapeutic development programs targeting IL-11.

AUTHOR CONTRIBUTIONS

RM wrote the manuscript and prepared the figures. TP wrote the manuscript. MG wrote the manuscript and supervised the studies. All authors contributed to the article and approved the submitted version.

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Modulation of Signaling Mediated by TSLP and IL-7 in Inflammation, Autoimmune Diseases, and Cancer

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Thymic Stromal Lymphopoietin (TSLP) and Interleukin-7 (IL-7) are widely studied cytokines within distinct branches of immunology. On one hand, TSLP is crucially important for mediating type 2 immunity at barrier surfaces and has been linked to widespread allergic and inflammatory diseases of the airways, skin, and gut. On the other hand, IL-7 operates at the foundations of T-cell and innate lymphoid cell (ILC) development and homeostasis and has been associated with cancer. Yet, TSLP and IL-7 are united by key commonalities in their structure and the structural basis of the receptor assemblies they mediate to initiate cellular signaling, in particular their cross-utilization of IL-7Rα. As therapeutic targeting of TSLP and IL-7 via diverse approaches is reaching advanced stages and in light of the plethora of mechanistic and structural data on receptor signaling mediated by the two cytokines, the time is ripe to provide integrated views of such knowledge. Here, we first discuss the major pathophysiological roles of TSLP and IL-7 in autoimmune diseases, inflammation and cancer. Subsequently, we curate structural and mechanistic knowledge about receptor assemblies mediated by the two cytokines. Finally, we review therapeutic avenues targeting TSLP and IL-7 signaling. We envision that such integrated view of the mechanism, structure, and modulation of signaling assemblies mediated by TSLP and IL-7 will enhance and fine-tune the development of more effective and selective approaches to further interrogate the role of TSLP and IL-7 in physiology and disease.

Keywords: cytokines, antagonist, agonist, protein-protein complex, therapeutic biologics, cytokine-receptor complex

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TSLP—EMERGING ROLE IN AUTOIMMUNE DISEASES AND CANCER

Over the last two decades TSLP has been extensively studied and known for its pivotal role in allergic conditions and involvement in chronic inflammatory diseases such as chronic obstructive pulmonary disease or inflammatory bowel disease (1–3). In recent years TSLP has additionally emerged as a novel molecular player in non-allergen induced conditions (4, 5). Together with broadening its pathophysiological profile these findings imply that the microenvironment of this pleiotropic cytokine might define the direction of its inflammatory response depending on the type of inflammation involved.

The development of IL-23-associated autoimmune disease psoriasis has recently been linked to overexpression of TSLP in keratinocytes from patient skin samples. It has also been demonstrated that serum levels of TSLP correlate to the severity of the disease (6, 7). Even though the role of TSLP in psoriasis is not completely resolved, TSLP has been reported to induce DC maturation and to drive to a DC-derived IL-23 production leading to the hypothesis that it could have a comparable role in other IL-23-driven autoimmune diseases (8). Furthermore, TSLP has been linked to rheumatoid arthritis (RA): increased TSLP levels in synovial fluid of patients with RA in comparison to those with osteoarthritis have been reported in several studies (9). Moreover, TSLP receptor (TSLPR) has also been found overexpressed in myeloid dendritic cells (mDCs) in synovial fluid of the RA patients. The engagement of TSLP in inflammatory arthritis is explained through TSLP-mediated priming of the mDCs and subsequent chemokine stimulation of CD4+ T cells proliferation leading to secretion of interferon γ (IFNγ), IL-17, and IL-4 (10). Additionally, several reports based on studies via mouse models and in humans provide further evidence of TSLP's possible involvement in the pathogenesis of different types of autoimmune disorders underlining a rising need to further interrogate the role of TSLP in relation to Th17 inflammatory response (11–13).

Thus, given such emerging evidence of the involvement of TSLP in the abrogation of Th1, Th9, and Th17 inflammatory responses and its influence on a range of immune cell lineages it does not come as a surprise that TSLP is becoming more intensively studied in the context of cancer (5, 8, 14). Nevertheless, the current view of the field regarding the role of TSLP in cancer has been divided in terms of tumor-progressive or tumor-protective effects depending on the type of cancer being studied. Studies focusing on tumors of hematopoietic and lymphoid tissues such as lymphoma and acute lymphocytic leukemia (ALL) reported TSLP as a tumor-progressive factor (15, 16). In addition, various genomic analyses detected chromosomal rearrangements and alterations of genes encoding TSLPR/CRLF2 in a large number of patients with B-cell precursor ALL (BCP-ALL), all of them having in common either an enhanced or constitutive expression of TSLPR leading to a signal boost resulting in resistance to therapy, high recurrence rate, and poor clinical outcome (17–19). Interestingly, TSLPR was also found overexpressed in 15% of B-ALL cases with no typical chromosome aberrations (20). Results from diverse experimental and clinical studies in diverse solid tumors—cervical, ovarian, pancreatic, or gastric cancer further imply an evident tumor-progressive role of TSLP in tumor microenvironment leading to the promotion of tumor angiogenesis and its growth and metastasis (21–27). On the other hand, both tumor-progressing and anti-tumor effects of TSLP have been demonstrated in diverse breast cancer studies (28–31). In contrast, skin and colon cancer studies reported TSLP-mediated anti-tumorigenic role hence emphasizing the urgency and importance of understanding this duality in the framework of developing suitable future therapeutics (32, 33).

GENETIC VARIATIONS IN IL-7 AXIS PLAY A ROLE IN BOTH AUTOIMMUNITY AND CANCER

Given that tight control of signaling mediated by IL-7 is essential to support and maintain immune homeostasis, it is not surprising that dysregulation of its stimulation leads to a disrupted lymphoid development and pathophysiology in different types of conditions including autoimmune diseases and cancer. Seeing that the absence of IL-7 mediated signaling leads to lymphopenia, a role of its regulation in autoimmune diseases could be implied (34). Several studies involving patients with multiple sclerosis (MS) and primary Sjögren's syndrome (pSS) demonstrate that both IL-7 and IL-7R are overexpressed in the cerebrospinal fluid and labial salivary glands, respectively, with these expression levels correlating to the severity of the disease (35–37). Increased susceptibility to autoimmune diseases such as MS, type 1 diabetes or RA have been linked to several single nucleotide polymorphisms in IL-7R gene loci (35, 38–40). In the case of MS, haplotypes in *IL7R* gene have been reported to lead to modulations in levels of soluble IL-7R which has also been upregulated in patients with pSS (41, 42). Based on additional extensive data from mice and human *in vitro* experiments, the pivotal involvement of IL-7 is more than evident. However, the mechanism of how the regulation of the IL-7 axis leads to increased susceptibility to autoimmune diseases still remains largely unclear, although the current mechanistic view suggests that activation of IL-7 signaling promotes the expansion of T cells and increased proliferation to self-antigens leading to predisposition to autoimmunity (43).

Whereas, IL-7 signaling is not as crucial in B-cell development in humans as it is in mice, cells from acute leukemia proliferate in response to IL-7 *in vitro* and have a corresponding expression of IL-7Rα (44). In addition, overexpression of IL-7Rα has recently been linked with relapse in pediatric B-ALL (45). IL-7Rα gene loci have been shown to carry gain-of-function mutations in a small fraction of patients with BCP-ALL with most of the mutations being associated with concurrent upregulation of TSLPR upregulation and ligand-independent activation of signaling (46). Involvement of the IL-7 signaling axis in the progression of T-ALL has been confirmed in extensive diverse studies showing stimulation of T-ALL cells with IL-7 and overexpression of IL-7Rα (47–52). This evidence is supported by identification of additional IL-7Rα gain-of-function mutations in T-ALL patients that lead to constitutive IL-7 independent receptor activation or else increased activation of the receptor resulting in increased IL-7 response (53, 54). Taking into account that 10% of T-ALL patients carry *IL-7Rα* mutations that have been linked to poor prognosis and risk in relapsed patients, IL-7Rα and its signaling pathways have emerged as logical therapeutic targets (55). Aside from strong evidence that IL-7 has anti-tumor effects, some studies indicate that IL-7 might enhance tumor-progression, for instance as suggested in studies focusing on non-small cell lung cancer cells (56). It is clear that further studies will be needed to better clarify the role of IL-7 in tumor-progression.

BIOLOGY, RECEPTOR ACTIVATION, AND SIGNALING OF TSLP AND IL-7

In response to pathogenic stimuli or mechanic injury, TSLP gets produced mainly by epithelial cells at barrier surfaces such as lung and gut, epidermal keratinocytes, and dendritic cells. However, we now know that TSLP has a much broader expression profile that extends to fibroblasts, macrophages, basophils, monocytes, and cancer cells (57–68). In addition to being involved in proliferation and differentiation of B-cell progenitors, TSLP expression and signaling leads to activation of immature DCs, CD4+ T-cell homeostasis and regulatory T-cell development through a coordinated cascade of immune and non-immune cells indicating its influence far beyond the widely acknowledged type 2 inflammatory responses (5, 61, 69–78).

TSLP mediates signaling by establishing a heteromeric complex involving TSLPR, a type I cytokine receptor, and IL-7Rα, a receptor also utilized by IL-7 (79, 80). TSLPR binds TSLP with high affinity ($K_D = 32$ nM), while IL-7Rα does not bind to TSLPR alone with measurable affinity. However, IL-7Rα can be recruited with high affinity ($K_D = 29$ nM) to the TSLP:TSLPR complex making this binary assembly a mechanistic prerequisite for effective signal transduction (81, 82). Of note, human TSLPR and IL-7Rα were shown to bear low, albeit measurable affinity (K_D 20 μM) for each other in the absence of TSLP, suggesting that preformed receptor-receptor interactions might play a role in the assembly of a TSLP-mediated complex under certain conditions (82). The ensuing dimerization of both receptor chains upon TSLP binding results in activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) leading to transcription of target genes and subsequent tightly coordinated immune responses (83–86) (**Figure 1**). Recently, tools have been developed exploiting the signaling properties of TSLPR for the screening and characterization of the activity of various types of cytokines and their receptors (87).

Similar to TSLP, IL-7 is predominantly secreted by non-lymphoid cells like keratinocytes and epithelial and stromal cells in lymphoid organs with highest expression levels being detected in thymus and lymph nodes. In contrast to several other members of IL-2 family cytokines, IL-7 is not produced by hematopoietic cells (88–93). In fact, it is now known that together with ILCs, hematopoietic cells express IL-7Rα and therefore play a role in lymphoid consumption and regulation of IL-7 availability (94). While the IL-7Rα on lymphoid cells regulates both TSLP and IL-7-mediated signaling, non-lymphoid cell types carrying IL-7Rα mediate only TSLP signaling (95). IL-7 signaling is essential for the development and homeostasis of T lymphocytes and several members of recently discovered ILC family, whereas its role in early development of B lymphocytes has been shown to be more substantial in mice than in humans (95–97).

IL-7 signals through a heterodimeric receptor complex consisting of IL-7Rα and the γc (98). The observed stepwise mechanism of the ternary assembly mediated by IL-7 is analogous to the one suggested for TSLP and γc family interleukins, whereby the formation of a high affinity IL-7:IL-7Rα constitutes a mechanistic requirement for the assembly of the signaling-competent ternary complex (**Figure 1**).

However, contrary to TSLP binding to TSLPR, binding of IL-7 to IL-7Rα has been proposed to proceed via biphasic binding kinetics manifested by two sets of on- and off-rate constants (99). The presence of such an unusual IL-7:IL-7Rα structural intermediate implied by the proposed model requires further confirmation by orthogonal biophysical approaches that may allow detection of conformational changes. Additional mechanistic considerations centering on the possibility of IL-7Rα homodimers and IL-7Rα-γc heterodimers in the absence of IL-7, have also been proposed: upon the presence of IL-7 a pre-associated IL-7Rα-γc heterodimer undergoes a rotation away from the cell surface bringing the C-termini of the IL-7Rα and γc within the distance allowing them to form an activating complex (100). A similar mechanism involving an IL-7Rα homodimer could serve as an explanation of the signaling effects that the gain-of-function mutations in T-ALL and B-ALL patients have independent of γc and IL-7 presence (46, 53). As an example, it has been shown that the S185C IL-7Rα mutation present in B-ALL patients leads to the formation of an additional disulfide bond between the two S185C IL-7Rα chains whereas the mutation of the cysteine to a glycine eliminates this effect (46). Results obtained from structural modeling of this interaction confirm the suggested mechanism by showing that the disulfide bond between the receptor chains allows the signal inducing proximity without the presence of a ligand (100).

Strikingly, whereas binding of IL-7 to non-glycosylated IL-7Rα shows medium affinity (low μM range) there is a dramatic decrease in the apparent K_D when binding the glycosylated IL-7Rα (low nM range) irrespective of glycan type or branching (99). Decoy IL-7Rα also plays an important role in regulation of IL-7 in immunity and in disease states (101). While the membrane bound IL-7Rα chain participates in signaling, soluble IL-7Rα competes with the membrane-bound receptor to eliminate excessive IL-7 thereby re-establishing its low levels as normally observed *in vivo* (102). Ligand-induced dimerization of IL-7 receptor leads to the activation of JAK-STAT, PI3-kinase, and MAPK/Erk signaling cascades and their respective responses (103–108).

STRUCTURAL BASIS OF TSLP-MEDIATED SIGNALING

Consistent with its annotation as a member of the IL-2 family of helical cytokines, structural studies by X-ray crystallography of human and mouse TSLP in complex with its cognate receptors revealed that TSLP adopts a four-helix bundle structure with alpha helices (αA, αB, αC, and αD) following an 'up-up-down-down' topology and six cysteine residues forming pairs of disulfide bridges (81, 82). Recently, evidence of two isoforms of human TSLP originating from the TSLP gene—a short and a long form—has been reported largely based on mRNA expression profiles (109). Given that it is currently unclear whether the short isoform of TSLP is translated into a biologically active protein, we will only be focusing on the well-established and biologically active long form of TSLP.

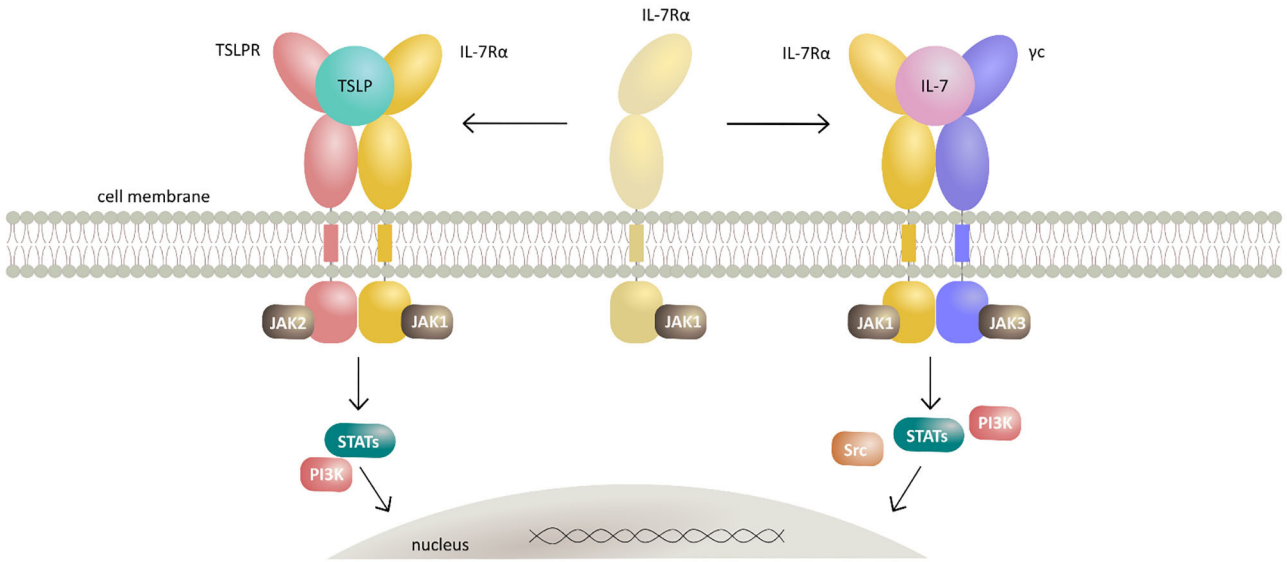

FIGURE 1 | Schematic representation of TSLP and IL-7 signaling mechanisms by their respective receptor heterodimerization upon cytokine binding. Cytokines TSLP and IL-7 both signal through heterodimeric receptors by sharing the IL-7Rα receptor chain. TSLP first interacts with the cognate TSLPR thus potentiating the recruitment of IL-7Rα and formation of extracellular ternary complex leading to activation of intracellular signaling by canonical JAK/STAT and PI3K pathways. Together with IL-7Rα and the γc, IL-7 forms a heterodimeric receptor complex resulting in activation of JAK/STAT, PI3K, and SRC pathways.

Intriguingly, human TSLP displays a unique structure among four-helix bundle cytokines manifested via a rather open helix-bundle core harboring a substantial internal void volume adjacent to a fully buried water molecule coordinated by three conserved residues (Trp148, Thr102, and Thr83). Furthermore, helix αA displays a substantial kink centered at about its midpoint that is hallmarked by a π-helical turn, a structural feature that has been linked to the enhancement of protein functionality (110). Complementary structural studies on unliganded human TSLP in solution by nuclear magnetic resonance established the flexibility of helix αA at its π-helical turn (82). The TSLP four-helix bundle is threaded by three loops, a *BC*-, *AB*-, and *CD*- loop, with the latter harboring a stretch of 7 basic amino acids (residues 125–131) the role of which remains unclear. However, it has been proposed that this segment encodes for a furin cleavage site and might provide a level of regulation for the secreted amounts of TSLP (82) (**Figure 2A**).

Based on high-resolution structural insights from the TSLP:TSLPR:IL-7Rα ternary complex, the attraction of TSLP to a cytokine binding homology region (CHR) in TSLPR (site I) is characterized by electrostatic complementarity between a positively charged surface on TSLP and negatively charged one on TSLPR (**Figure 2B**). Formed binary complex TSLP:TSLPR has a calculated negative electrostatic potential which supports subsequent binding of IL-7Rα having a positive electrostatic potential. Part of residues involved in TSLP:TSLPR interactions are located in the C-terminal part of αD helix (residues 142–158) and *AB*-loop region (residues 60–69) undergoing significant conformational changes for obtaining the bound state. The *AB*-loop is simultaneously offering a physical link to the αA helix important for interactions with IL-7Rα (site II) thus being a mediator of priming TSLP to recruit the IL-7Rα after forming TSLP:TSLPR complex which then facilitates the positioning of the αA helix providing a necessary entropic advantage for the formation of a T-shaped ternary complex. Besides residues in αA helix, hydrophobic interface of IL-7Rα furthermore interacts with several exposed residues in αC helix of TSLP (**Figure 2C**). Compact interaction of TSLPR and IL-7Rα in their membrane proximal region (site III), also known as the stem region, is characterized by electrostatic interactions and close van der Waals contacts. Interactions in site III have been proven to contribute to the effective TSLP-mediated signal transduction (82) (**Figure 2D**).

STRUCTURAL BASIS OF SIGNALING ASSEMBLIES MEDIATED BY IL-7

Just like TSLP and other cytokines of the IL-2 family, IL-7 has four helices (αA-αD) adopting the "up-up-down-down" topology. While homology models had predicted the presence of three disulfide bonds, electron density of crystal structures obtained was too weak at the N-terminus and the end of helix αC where third disulfide bond was predicted resulting in tracing only two of the cysteine pairs (99, 112). Structural data shows burial of the only tryptophan residue of IL-7 in the hydrophobic core of the helix bundle, consistent with mutagenesis studies that linked this position to the proper folding of IL-7 (99, 113, 114). As in TSLP, helix αA of IL-7 comprises a π-helical turn of six residues stabilizing the IL-7:IL-7Rα interaction. This interface also includes contacts with αC residues and is generally characterized by hydrophobic, van der Waals and few intermolecular polar interactions (**Figures 2E,F**).

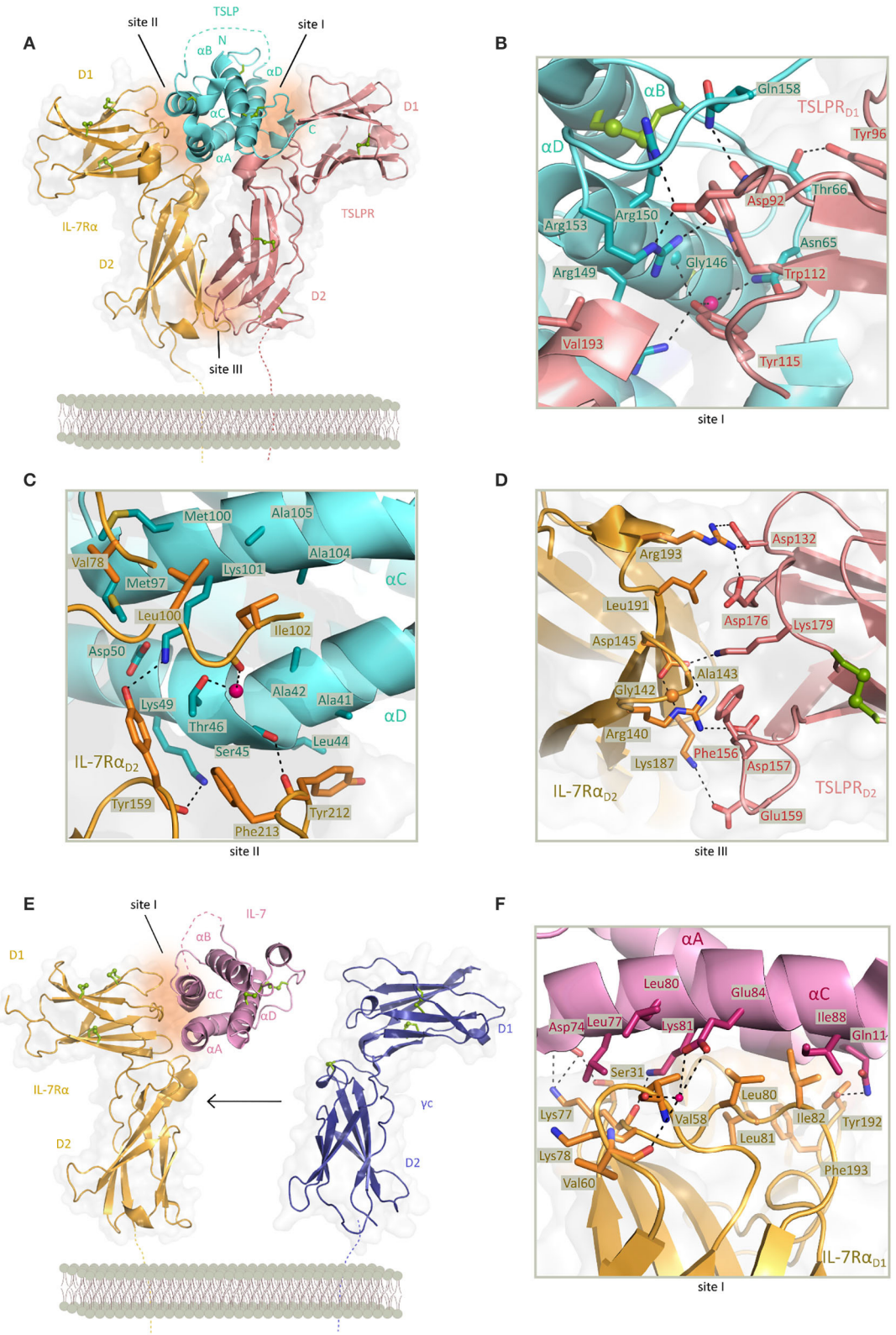

FIGURE 2 | Structure of TSLP and IL-7 receptor complexes and structural close up view of the cytokine-receptor contact interfaces. **(A)** View of the determined X-ray structure for the TSLP:TSLPR:IL-7Rα ternary complex. TSLP is shown in aquamarine cartoon representation with four helices marked αA-αD and the disordered CD

(Continued)

FIGURE 2 | loop region is shown as a dashed aquamarine line. The extracellular regions of TSLPR (salmon pink) and IL-7Rα (bright orange) each comprising of two FnIII-like domains D1 and D2 are shown as cartoons on a transparent gray surface representation. Disulfide bridges are represented by green spheres. Regions contributing to protein-protein contact are named site I (TSLP:TSLPR), site II (TSLP:IL-7Rα), and site III (TSLPR:IL-7Rα) and represented by dark orange surfaces. [PDB 5J11, (82)] **(B)** Detailed representation of the TSLP:TSLPR interface (site I). **(C)** Detailed representation of the TSLP:IL-7Rα interface (site II). **(D)** Detailed representation of the TSLPR:IL-7Rα interface (site III) viewed from the membrane-proximal side. In **(B–D)** interface residues are shown as sticks and hydrogen bonds and salt bridges are indicated with a dashed line. Water molecule is depicted as a pink sphere. **(E)** View of the X-ray structure for the IL:IL-7Rα binary complex. IL-7 is shown in pink cartoon representation with four helices marked αA-αD and the second crossover loop shown as a dashed pink line. The extracellular regions of IL-7Rα (bright orange) and γc (purple) are comprising of two FnIII-like domains D1 and D2 shown as cartoons on a transparent gray surface representation. The γc from the IL4 ternary complex has been depicted apart from the IL:IL-7Rα complex as it has not been co-crystallized with the binary complex [PDB 3DI2, 3BPL, (99, 111)]. Disulfide bridges are represented by green spheres. Region contributing to IL-7:IL-7Rα contact is named site I and represented by dark orange surface. **(F)** Detailed representation of the IL-7:IL-7Rα interface (site I). Interface residues are shown as sticks and hydrogen bonds and salt bridges are indicated with a dashed line. Water molecule is depicted as a pink sphere.

While other cytokines that signal via γc family receptors interact with their specific receptor chain via a larger buried surface consisting of predominantly polar residues, the IL-7:IL-7Rα interface is not only less extensive but also more hydrophobic (99). Interestingly, the TSLP-bound conformation of IL-7Rα is highly similar to the one observed in IL-7:IL-7Rα and IL-7Rα employs a nearly identical set of residues to bind each of the cytokines. These unique structural features shared between TSLP and IL-7 allow a predominantly hydrophobic interaction with IL-7Rα providing a rationale for duality and degeneracy of signaling via IL-7Rα (82). Nevertheless, the structure of the IL-7:IL-7Rα complex does readily explain the large differences in the affinity of IL-7 to the glycosylated and non-glycosylated forms of IL-7Rα as all candidate glycosylation sites are not in close proximity to the IL7:IL7Rα interface (99).

So far, there has been no structural data for the ternary IL-7:IL-7Rα:γc assembly, which would fill a large void in our understanding of the extracellular signaling assembly mediated by IL-7. Besides comparing structural and mutagenesis data coming from other cytokine-receptor binary and ternary assemblies in γc receptor family, efforts have been made to model this interaction using the IL-7:IL-7Rα binary complex and γc structures extracted from other complexes. These approaches suggested the critical involvement of a disulfide bond and involvement of several key residues on γc for the formation of a ternary complex. Although the models propose the canonical engagement of helices αA and αD in binding the γc receptor, the proposed models also suggest the IL-7:γc interface will differ substantially from other γc:interleukin interactions. The differentiation of cytokines by γc is additionally facilitated by angular displacement of the helices to form a distinct binding epitope. Indeed, superpositon of IL-2, IL-4, and IL-21 as observed in their receptor complexes onto IL-7 revealed a substantial difference in the inter-helical orientation (99). Superpositions of fibronectin type III (FnIII) domains of IL-7Rα and IL-4Rα binary and ternary complexes onto IL-2Rβ show differences in angular geometry between the domains resulting in steric clashes and a lack of availability of helices to contact γc receptor. This suggests that it requires another conformation and a more drastic elbow angle between the two FnIII domains to adequately form IL7:IL-7Rα:γc (99, 100).

STRATEGIES TO MODULATE SIGNALING MEDIATED BY TSLP AND IL-7

In light of the tremendous importance of cytokines in health and disease, recent efforts have focused on harnessing structural and functional data interrogating cytokine-receptor interactions and functionality toward the development of potent antagonists and agonists that can modulate cytokine-mediated signaling (115) (**Figure 3**). Such modulators would be expected to have distinct modes of action compared to already available inhibitors known to target intracellular portions of cytokine receptors or specific intracellular signaling components downstream of cytokine-dependent receptor activation (116, 117). Nevertheless, extracellular portions of cytokine receptors and the activating cytokines are attractive targets in their own right. In this context, modulation of their activity could be achieved by both biologicals and non-biologicals engineered to bind in either orthosteric or allosteric fashion. Preventing cytokine action in such a context has mostly been achieved by neutralizing antibodies developed against either the cytokine or one of its receptors or by using soluble ectodomains of the cytokine receptors as molecular decoys (101, 118–121). These approaches do not necessarily require structural insights and are therefore favorable in targeting cytokine/receptor interactions when no such data is available. To this end, promising alternatives are in the form of Fc-fused receptors or cytokine traps comprising fusions of the cognate receptor ectodomains via flexible linkers (82, 122–124). Cytokine-derived antagonists present another approach in which structural information together with the mechanistic insights are crucial for establishing the critical cytokine-receptor interaction determinants to arrive at cytokine variants having desired modulatory characteristics and potency (115). In the case of heterodimeric receptor complexes, this approach is based on the engineering a cytokine such that it can only bind one of the receptor chains with high affinity thereby being unable to recruit the second receptor to form a signaling complex with IL-4 antagonist as one of the best-known examples (113, 125). Such a strategy led to the design of muteins with remarkable new properties: for instance, an IL-4 antagonist (Pitrakinra^{TM}) is able to block both IL4/IL-13 receptor assembly formation, an IL-2 superkine where the engineered new property bestows a higher affinity for IL-2Rβ chain, or the most recently reported

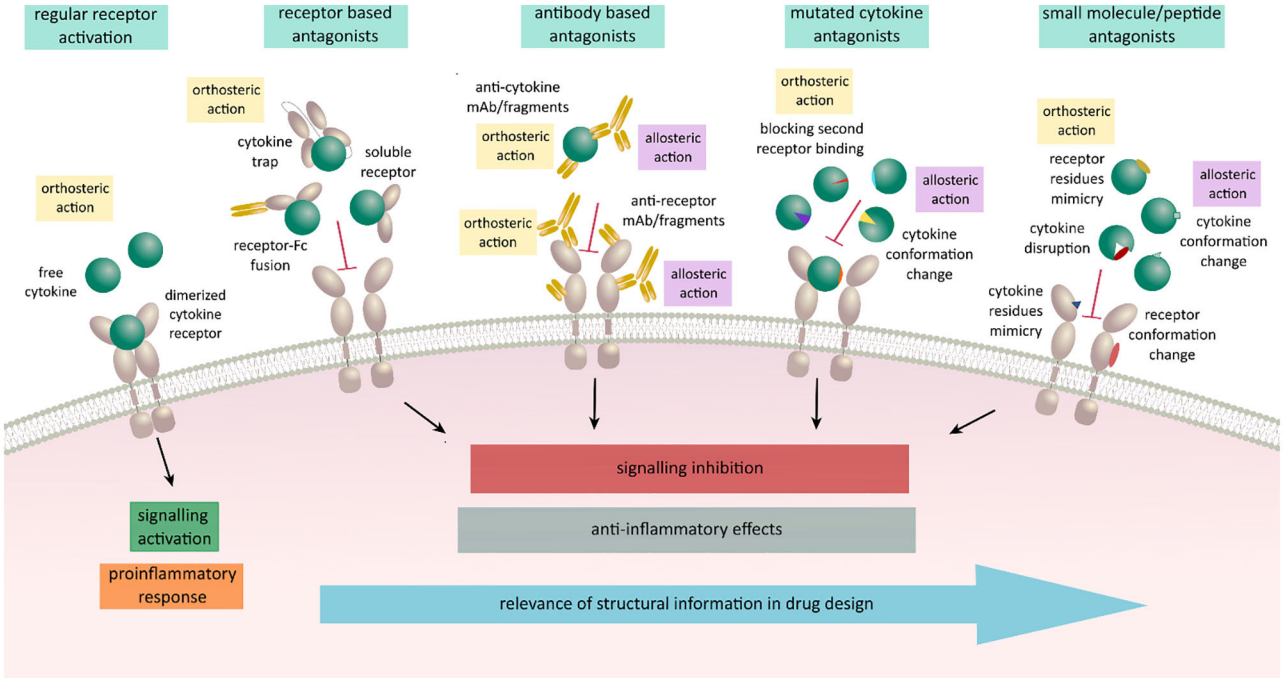

FIGURE 3 | Schematic representation on various antagonistic strategies used for regulation of cytokine signaling acting in an allosteric or an orthosteric fashion. Receptor activation leads to signaling activation upon cytokine binding and receptor dimerization resulting in a proinflammatory responses and various disease states. These responses can be meliorated by the use of biologicals engineered to orthosterically or allosterically block the receptor activation. Receptor based antagonists act in an orthosteric fashion by blocking the expressed cytokine and preventing it to bind to its respective ligand. Antibody based antagonists consist of either full antibodies, Fab fragments, scFv, or nanobodies against the cytokine or its receptor. They can either act orthosterically at the binding site and prevent the binding of cytokine to its specific receptor or allosterically by binding outside of the binding site affecting the conformation and resulting in the lack of binding ability of either cytokine or the receptor. Mutated variants of cytokines are designed to act in both ways by keeping the ability to bind to their specific cytokine receptor chain and losing the affinity to the second receptor chain due to selected mutations. Small molecule and peptide antagonists design is based on either mimicking cytokine or receptor interaction residues, disrupting the proper folding of the protein or binding outside of the interaction sites inducing a conformation change resulting in blocking of receptor activation and signal transduction.

neoleukins designed to play into the functional dichotomy of IL-2 and IL-15 (126–128).

Peptide-based inhibitors can have high structural similarity to fragments of the target proteins and in that way mimic protein-protein interactions crucial for signaling. Their additional advantage is simple synthesis and possibility to modify their peptide sequences using diverse functional groups (129, 130). Together with small molecule inhibitors, such inhibitors focus on targeting hot spots and binding gaps at cytokine-receptor interfaces and modulate their activity as demonstrated for IL-2 and TNF and suggested for IL-18 (131–134). Although challenging to develop, potent small molecules inhibitors are considered to offer substantial advantages over biologics or protein-based modulators, including oral and topical administration (135).

MODULATORS OF TSLP SIGNALING

Anti-TSLP monoclonal antibody (mAb) Tezepelumab (AMG157/MEDI9929) has first been reported in 2014 and is to date the most prominent and advanced inhibitor of TSLP-mediated signaling in the context of allergic inflammatory disorders and the only TSLP-linked antagonistic candidate that is currently in phase III clinical trial in patients with severe asthma (136–138). The neutralizing effects of this antibody suggested that it recognizes an epitope in the domain of cytokine responsible for binding of TSLPR chain. Indeed, the X-ray structure of $AMG157_{Fab}$ fragment in complex with TSLP confirmed this by showing that complementarity determining regions (CDRs) of the variable heavy chain domain of Fab fragment interact with the AB-loop region and C-terminal region of helix D, while the light chain fragment does not interact with TSLP. In the same time, the other side of TSLP helical bundle involved in binding IL-7Rα remains available (82) (**Figure 4A**).

The most recent publication covering phase II trial in adults with a history of asthma exacerbations and uncontrolled asthma reports the study being conducted by subcutaneous Tezepelumab application over 52 weeks. Patients who were previously receiving asthma controllers and received Tezepelumab therapy showed rate reductions of clinically significant asthma exacerbations of at least 62% compared to the patients receiving placebo independent of baseline eosinophil counts (140, 141). Ongoing phase III clinical trial includes further mechanistic and

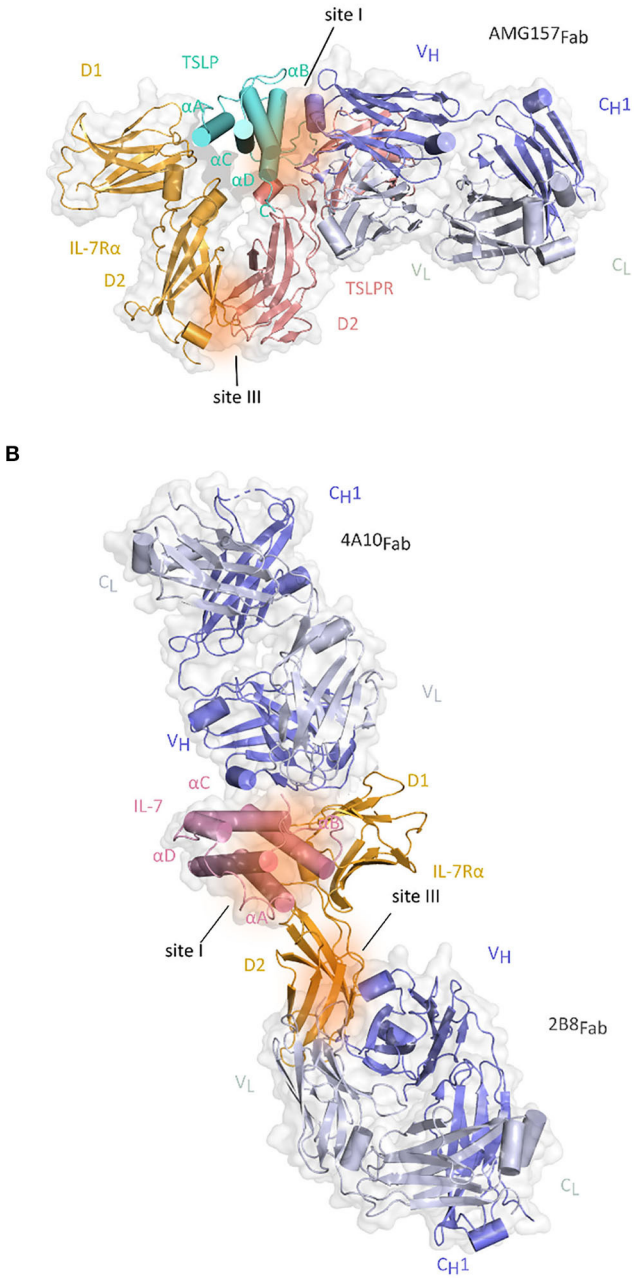

FIGURE 4 | Structure of Fab fragments of the antibodies developed against TSLP and IL-7Rα together with their respective cytokine-receptor complexes. **(A)** Cartoon representation of the TSLP:TSLPR:IL-7Rα:AMG157$_{Fab}$ complex by superposition of the TSLP:TSLPR:IL-7Rα and TSLP:AMG157$_{Fab}$ based on structural alignment of the two TSLP structures [PDB ID:5J11, 5J13 (82)]. TSLP is shown in aquamarine cartoon representation with its four helices marked αA-αD. The extracellular regions of TSLPR (salmon pink) and IL-7Rα (bright orange) each comprising of two FnIII-like domains D1 and D2 are shown as cartoons on a transparent gray surface representation. Regions contributing to protein-protein interactions are represented by dark orange surfaces and named site I (TSLP:TSLPR) and site III (TSLPR:IL-7Rα). V_H and C_{H1} fragments of AMG157$_{Fab}$ are colored in violet blue and the V_L-C_L fragments in white blue and shown as cartoons on a transparent gray surface representation. **(B)** Cartoon representation of the IL-7:IL-7Rα:4A10$_{Fab}$:2B8$_{Fab}$ *(Continued)*

FIGURE 4 | by superposition of IL-7Rα:4A10$_{Fab}$ and IL-7Rα:2B8$_{Fab}$ and IL-7:IL-7Rα based on structural alignment of IL-7Rα chains [PDB ID 3DI2, 6P50, 6P67 (99, 139)]. IL-7 is shown in pink cartoon representation with four helices marked αA-αD on a transparent gray surface representation. The two FnIII-like domains (D1 and D2) of IL-7Rα (bright orange) are shown as cartoons. Regions contributing to cytokine-receptor interactions are represented by dark orange surfaces and named site I for IL-7:IL-7Rα interface and site III for TSLPR:IL-7Rα and plausible γc interaction site. 4A10$_{Fab}$ and 2B8$_{Fab}$ are depicted as described for AMG157$_{Fab}$ in **(A)**.

long-term safety trials with the focus on both adult and adolescent patients with severe asthma considering previous or current treatments with different combinations of asthma controller medications. Initial safety and preliminary clinical activity of Tezepelumab in AD patients were evaluated in phase I study and showed no immunogenicity and good tolerance (142, 143). Phase IIa study in patients with moderate to severe AD receiving the antibody subcutaneously in combination with topical corticosteroids showed improvements in severity of the disease yet they were not statistically significant (144, 145). A Phase IIb study is designed to evaluate the safety and efficacy of Tezepelumab as a monotherapy and adjunct therapy in patients with moderate to severe AD and is currently in the patient recruiting stages (146). Confirmed evidence of efficacy of this antibody in treatment of a possibly broad population of asthma patients together with potential therapeutic benefit in patients with AD puts Tezepelumab on the map of promising therapeutics in inflammatory and possibly other diseases as well. Furthermore, the antibody-based inhibitor CSJ117 has been developed in a form of an inhalable Fab antibody fragment against TSLP. It has been used in a recently completed Phase I clinical trial in asthma patients with mild atopic asthma to access the safety, tolerability, pharmacokinetics, and pharmacodynamics of the inhaled agent (147). Moreover, novel human single-chain variable fragment (scFv) was also developed against human TSLP and selected from a fully human antibody library. ScFv29 is shown to bind to human TSLP in competition with the TSLPR. As it can also bind to murine TSLP, *in vitro* experiments performed on mouse derived mDCs showed that ScFv29 reduces the maturation rate of DCs making it one of the potential neutralizing candidates of this system (148). With the aim of increasing the therapeutic effects in asthma patients that have been shown to co-express TSLP and IL-13 and contribute to the severity of the disease Venkataramani and colleagues have developed novel bispecific anti-TSLP/IL-13 antibodies that are either monovalent bispecific (Zweimabs) or bivalent bispecific (Doppelmabs) and can simultaneously inhibit the signaling of both cytokines (149, 150). Binding and functional data show stronger affinity of the bispecific antibodies in comparison to their parental monospecific ones and inhibitory effects in DCs assays demonstrating the potency of such dual targeting (151) (**Table 1**).

The inhibitory mAb RG7258 considered as a potential therapeutic in allergic disorders has been developed against TSLPR. It cross reacts with the cynomolgus monkey TSLPR and inhibits TSLP-induced responses *in vitro*. Furthermore, in

TABLE 1 | Overview of the current modulators of TSLP signaling in preclinical studies and clinical trials.

Signaling modulator	Target	Preclinical studies	Target disease	Clinical trials	References
Human IgG2λ mAb Tezepelumab MEDI9929 AMG157	TSLP	BAF3-TSLPR cells Human blood DCs	Inflammatory allergic diseases	Phase I asthma Phase I asthma adolescents Phase I healthy subjects Japan Phase IIb asthma Phase II CASCADE Phase I AD Phase IIa ALLEVIAD Phase I/Phase II cat allergy Phase III NAVIGATOR Phase III PATH-HOME Phase I PATH-BRIDGE	(136, 137) (152) (153) (140, 141) (154) (142, 143) (144, 145) (155) (138) (156) (157)
mAb IgG2λ Fab fragment CSJ117	TSLP		Inflammatory allergic diseases	Phase I asthma	(147)
Human scFv ScFv29	TSLP	Mouse derived mDCs	Inflammatory allergic diseases		(148)
Bispecific mAbs Zweimabs and Doppelmabs	TSLP and IL-13	Human blood DCs	Inflammatory allergic diseases		(151)
Humanized IgG1 mAb RG7258	TSLPR	Human DCs Human mast cells Ascaris-sensitive cynomolgus monkey model	Inflammatory allergic diseases	Phase I asthma (discontinued)	(158, 159)
Human IgG1 mAb ASP7266	TSLPR	Human and monkey peripheral white blood cells, human mDCs, cynomolgus monkeys, ascaris-sensitive cynomolgus monkeys model	Inflammatory allergic diseases	Phase I asthma Japan (discontinued)	(160, 161)
mAb 1E10	TSLPR	BaF3-TSLPR cells $TSLPR^+$ BCP-ALL LTCs	Leukemia BCP-ALL		(162, 163)
TSLP cytokine traps	TSLP	HEK293T cells, human blood DCs	Inflammatory allergic diseases		(82)
Small molecule Baicalein	TSLP	HMC-1 cells, HDM-induced mouse model of airway inflammation, OVA-induced mouse model of pulmonary eosinophilia	Inflammatory allergic diseases		(164)

the ascaris-sensitive cynomolgus monkey model mAb RG7258 successfully reduced inflammation and bronchoconstriction (158). Another anti-TSLPRα antibody ASP7266 is able to inhibit TSLP signaling in peripheral white blood cells and mDC-mediated differentiation of naive CD4+ T cells into mature T cells *in vitro*. Intravenous administration to monkeys effectively blocked CCL17 mRNA expression in peripheral blood cells and suppressed skin allergic reactions in sensitized cynomolgus monkeys (160). However, phase I clinical trial in asthma patients in Japan has been discontinued for undisclosed reasons (161). One additional mAb generated against extracellular domain of TSLPR was reported as one out of two hybridoma clones that showed antagonistic properties toward TSLP without affecting IL-7/IL-7R signaling (162). A subsequent study by the same group tested the antagonistic potential of 1E10 clone in the context of ALL and showed that blocking of TSLPR represses proliferation and STAT activity in $TSLPR^+$ BCP-ALL long term cultures (LTC) making it a potential therapeutic option for subset of BCP-ALL patients whose lymphoblasts express TSLPR (163) (**Table 1**).

The physiological role of soluble versions of cytokine receptor ectodomains as modulators of cytokine signaling in physiology and disease, and the fact that a naturally existing soluble TSLPR has not yet been identified, inspired the employment of the TSLPR ectodomain or its engineered variants as potential neutralizers of TSLP-mediated signaling. In a first study with such focus, a fusion protein TSLPR-Ig was designed by fusing the ectodomain of murine TSLPR with a murine IgG2a Fc tail. The effect of blocking TSLP signaling by murine TSLPR-Ig was tested in TSLP-activated murine DCs *in vitro* where it reduced the expression of CD40, CD80, and CD86. Additionally, local administration of murine TSLPR-Ig into the airways of asthmatic mice before sensitization suggested altering of the function of pulmonary DCs critical in Th2-mediated allergic disorders demonstrating that this blocking strategy could indeed be beneficial for treatment of asthma (165). Another

validation of using TSLPR complex ectodomains as inhibitors was demonstrated by utilizing both soluble TSLPR and IL-7Rα ectodomains in an equimolar mixture in a cellular STAT5 activity assay confirming their dose-dependent neutralizing effect. Arguably, the most potent approach to date situates in the employment of receptor fusion proteins, termed TSLP-traps, featuring tandem fusions of the TSLPR, and IL-7Rα ectodomains using a flexible $(Gly\text{-}Gly\text{-}Ser)_{20}$ linker. Notably, TSLP-trap1 showed a 250-fold higher affinity to TSLP when compared to the unlinked receptor ectodomains and a comparable affinity and binding kinetics to TSLP as both Tezepelumab mAb and its corresponding Fab fragment. A similar binding profile has been reported for TSLP-trap2. Competition assays in HEK293T cells showed that both TSLP-trap variants have about 1000-fold higher inhibitory potency over equimolar mixtures of unlinked soluble ectodomains and could outperform the inhibition of STAT5 signaling by 20–30-fold in comparison to the most potent anti-TSLP agent Tezepelumab and its Fab fragment. These intriguing data were further supported by evidence of both TSLP-trap variants having no effect on IL-7 function and being able to significantly block TSLP-driven DC-activation with the same efficacy as Tezepelumab (82). Thus, TSLP-traps represent promising novel biologicals with outstanding neutralizing potency.

The advent of high-resolution crystal structures of mouse and human TSLP:TSLPR ternary complexes (81, 82) offered the long missing structural blueprints to inspire the development of small molecule inhibitors targeting the TSLP:TSLPR interaction as a new therapeutic strategy. Van Rompaey and collaborators identified the first fragments to inhibit the TSLP:TSLPR interaction by a combined virtual—*in vitro* screening approach. The procedure consisted of an extensive *in silico* analysis of the structural data and evaluation of possible hot spots, screening of two commercially available fragment libraries followed by docking the hits to TSLPR, two-stage biological screening for further selection of the fragments and finally molecular dynamics to explore the binding pathway and model of fragment binding. This approach provided a proof-of-concept for the use of fragments in the modulation of TSLP signaling (166). Another study explored the potential of peptide-derived inhibitors designed based on amino acid sequences from murine TSLP:TSLPR structure that was the only structure available at the time. Solid-phase peptide synthesis was used to generate 16 peptides by mimicking epitopes of two TSLP:TSLPR interaction sites and resulted in three peptides capable of TSLP inhibition at submillimolar concentrations (167). The most recent report has focused on a flavonoid representing a major component of *S. baicalensis*, Balcalein, that was identified as the first small molecule inhibitor of TSLP-mediated signaling. Based on *in vitro* confirmation that the compound blocks TSLP:TSLPR interaction in a dose-dependent manner, *in vivo* studies in both HDM-challenged and OVA-sensitized mice resulted in reduced number of eosinophils in treated mice. Further chemical modeling led to a synthesis and identification of compound 11a, a biphenyl flavanone analog, which is considered the most advanced human TSLP inhibitor in this series of tests and characterized by moderate inhibition and good water solubility (164).

Efforts to identify TSLP antagonists for both murine and human cytokine have been made by analyzing TSLP:TSLPR and TSLP:IL-7Rα interaction sites. The most promising murine TSLP variant had one-point mutation, I37E, and showed high-affinity binding to TSLPR but no STAT5 activity in a cellular based assay (81, 82). From the selection of generated human mutants, a double mutant carrying S45R/T46R mutations residues at the TSLP:IL-7Rα interface in site II showed an unaffected ability to bind TSLPR and reduced STAT5-signaling in comparison to wt TSLP. From the selected mutants located in site I at the TSLP:TSLPR interface all of them reduced STAT5 signaling, with R149S/R150S double mutant having the most pronounced effect (82). In spite of not yielding a potent antagonist, those functional interrogations did demonstrate the importance of TSLP residues identifying hot spots that could be considered in further attempts in antagonist design.

MODULATORS OF IL-7 SIGNALING

Targeting of IL-7:IL-7Rα for therapeutic development in autoimmune diseases has mainly focused on the development of monoclonal anti-IL-7Rα antibodies which could be beneficial by blocking IL-7Rα and subsequently attenuating the action of effector T cells but retaining the T_{reg} activity (168).

Application of anti-IL-7Rα mAb 28G9/Ab1 in mice with autoimmune encephalomyelitis (EAE) was effective in reducing disease activity and severity (169). In non-obese diabetic mice, treatment with 28G9 delayed the progression of T1D before onset and reversed the newly onset diabetes (170). *Ex vivo* studies in cynomolgus monkeys showed a decreased STAT5 phosphorylation in both CD4+ and CD8+ T cells of blood samples after treatment (171). Another mAb (PF-06342674/RN168) developed by Pfizer has completed a phase I clinical trial in healthy volunteers. While the MS clinical trial was terminated by Pfizer themselves, the T1D phase I clinical trial evaluated the safety and tolerability of multiple SC doses in type 1 diabetes patients and showed that certain dose of mAb selectively inhibits survival and activity of memory T cells while preserving naive T cells and T_{reg} (172). Additional model study of its pharmacokinetics showed that it has a 20-fold more potent inhibitory effect on T_{EM} cells relative to T_{reg} cells at a similar dose confirming the implication that these effects could serve to eliminate pathologic T cells in autoimmune diseases (173) (**Table 2**).

Humanized Fc-disabled anti-IL-7Rα mAb, known under GSK261896, was well-tolerated in phase I clinical trial in healthy volunteers. It blocked IL-7 receptor signaling upon full target engagement, increased circulating IL-7 and soluble IL-7Rα, however showed no impact on peripheral T cell subsets or levels of other inflammatory cytokines (177). Phase I clinical trial in relapsing remitting MS patients got terminated because of the misrepresentation of preclinical data while the phase II trial in pSS patients was withdrawn resulting in no ongoing trials for this agent at the moment (180) (**Table 2**).

Two of the most recently developed anti-IL-7Rα antibodies might both potentially benefit patients with T-ALL. Akkapeddi

TABLE 2 | Overview of the current modulators of IL-7 signaling in preclinical studies and clinical trials.

Signaling modulator	Target	Preclinical studies	Target disease	Clinical trials	References
Human IgG1 mAb Ab1 28G9	IL-7Rα	Mice with EAE, mouse model T1D, cynomolgus monkeys	Autoimmune diseases		(169–171)
Humanized IgG1 mAb PF-06342674 RN168	IL-7Rα		Autoimmune diseases	Phase I healthy volunteers Phase Ib T1D (completed) Phase I MS (terminated)	(174) (172, 175) (176)
Humanized Fc-disabled mAb GSK2618960	IL-7Rα	No data publicly available (GSK)	Autoimmune diseases	Phase I healthy volunteers Phase I RRMS (terminated) Phase IIa pSS (withdrawn)	(177, 178) (179) (180)
Fully human IgG1 mAb B12 Antibody drug conjugate B12-MMAE	IL-7Rα	Ba/F3 and D1 cell lines, T-ALL cell lines, primary human T-ALL cells, NK-cells, Rag1–/– mice in combination with D1 cells and T-ALL cells	Leukemia (T-ALL)		(181)
Chimeric FAb human IgG1 4A10 2B8	IL-7Rα	D1 cell line, primary human T-ALL cells, Rag1–/– mice in combination with patient derived xenografts (PDX) cells	Leukemia (T-ALL)		(139)
rIL-7 CYT107		C57BL/6, c57BL/6-L5.1, BALB/c mice, CD1 mice	HIV, sepsis, Hematopoietic stem cell transplantation (HSCT), cancer	Phase I/II solid tumors Phase II sepsis IRIS-7-B Phase II cancer Phase I HSCT Phase I HIV Phase II HIV ERAMUNE-01 Phase I HIV INSPIRE Phase I cancer Phase II cancer ELYPSE-7 Phase I/II HCV ECLIPSE 1	(182) (183, 184) (185) (186, 187) (188, 189) (190, 191) (192) (193) (194, 195) (196) (197–199)
rIL-7 with hybrid human Fc IL-7-Fc NT-I7 GX-I7 Efineptakin alfa Hyleukin		Mice with syngeneic tumor graft, cynomolgus monkeys, BALB/c mice, C57BL/6 mice, and DO11.10 T cell receptor (TCR) transgenic mice, human colon adenocarcinoma xenograft mice	Cancer	Phase I healthy volunteers Phase I HPV	(200) (201) (202–204)

and colleagues reported a fully human IgG1 mAb, termed B12, developed against wildtype and several mutant IL-7Rα carrying insertions or single amino acid substitutions. A simulation of the structure of B12 in complex with the non-glycosylated IL-7Rα ectodomain indicated that the binding epitope is distinct from the interaction interface with IL-7. Based on the known structure of IL-7:IL-7Rα structure, IL-7 is positioned at the region connecting the D1 and D2 domains of the receptor, which implies that B12 could be located on the opposite side. B12:IL-7Rα binding interface is mostly hydrophobic with few van der Waals interactions and hydrogen bonds. B12 is able to inhibit IL-7-dependent and mutant-dependent IL-7R-mediated signaling and induce leukemia cell death. It promotes NK-mediated T-ALL cytotoxicity *in vitro*, delays T-cell leukemia development *in vivo* reducing tumor burden and promoting mouse survival and sensitizes T-ALL cells to treatment with dexamethasone inducing leukemia cell death. Those favorable effects were meliorated by B12-mono-methyl auristatin E (MMAE) antibody–drug conjugate (ADC) that is able to kill primary and patient-derived xenograft T-ALL cells more efficiently than B12 alone (181). The possibilities of ADCs in combination with IL-7R have previously been considered after confirming the involvement of IL-7 signaling in steroid-resistance when addressing the treatment of autoimmune diseases and cancers. The developed anti-murine IL-7R antibody conjugated with the compound SN38 showed strong anti-tumor effects against parental and steroid-resistant malignant cells, while the antibody-MMAE conjugate suppressed the inflammation in the mouse autoimmune arthritis model suggesting this approach as a possible novel alternative to steroid therapy (205). Furthermore, second study by the same group of collaborators that generated B12 shows that the two newly designed chimeric mAbs 4A10, and 2B8 recognize two separate epitopes on IL-7Rα based on the crystal structure of the $4A10_{Fab}$:IL-7Rα:$2B8_{Fab}$ complex. This structure reveals that $4A10_{Fab}$ interacts with the periphery of epitopes responsible for binding IL-7 and that $2B8_{Fab}$ binds close to the membrane region of the IL-7Rα, where TSLPR and the predicted γc binding sites would be situated (**Figure 4B**). Binding of $4A10F_{ab}$ to the extracellular portion of IL-7Rα has been shown to be 9-fold tighter than the

binding of $2B8F_{ab}$ with both K_D values in the low nM range compared to the binding properties of the mAbs to IL-7Rα on human lymphocytes. Moreover, these mAb chimeras inhibit IL-7R signaling at low IL-7 concentrations, mediate antibody-dependent cell mediated cytotoxicity *in vitro* and are effective in controlling established and relapsing leukemia *in vivo* (139) (**Table 2**).

Due to the pleiotropic nature of the biological activity of IL-7 and its central role in T-cell development, recombinant IL-7 (rIL-7) has been extensively tested in another, agonistic modulating frame. Indeed, multiple preclinical studies have confirmed that it could have therapeutic applications due to its potent immunorestorative and enhancing effect in immunotherapy and target multiple immunodeficiency conditions (206).

When used as an adjuvant in immunotherapy in sepsis patients with septic shock and severe lymphopenia, rIL-7 administered intramuscularly caused a 3- to 4-fold increase in absolute lymphocyte counts, reversed the marked loss of CD4+ and CD8+ immune effector cells and increased T cell proliferation and activation thus restoring adaptive immunity. As a novel approach in the treatment of patients with sepsis, another phase II study is planned in the future based on intravenous administration of rIL-7 (183, 184) (**Table 2**). Furthermore, patients suffering from infectious diseases may benefit from rIL-7 therapy given in a combination with antiviral drugs (188–192, 207), while patients who have received rIL-7 after hemopoietic stem cell transplantation (HSCT) showed T-cell recovery, implying a possibility of a lower risk of subsequent infection and relapse (186, 187) (**Table 2**).

In the context of cancer treatments rIL-7 has been used in clinical trials in patients with diverse types of tumors. A first study in humans was done in patients with metastatic melanoma and sarcoma and showed the ability of this cytokine to increase the number of $CD4^+$ and $CD8^+$ lymphocytes and decrease in the percentage of $CD4^+$ T-regulatory cells suggesting its role in treating lymphopenia. At the same time, this study showed that the non-glycosylated variant of IL-7 elicits a low titer of binding antibodies and could lead to potential side effects in higher doses, suggesting the advantage of IL-7 produced in eukaryotic systems (197). A Phase I study in patients with solid tumors has been completed and proved tolerance and rIL-7 potency resulting in rejuvenated circulating T-cell profile with increase in overall naive T cells but a decreased T_{reg} number making this effect opposite from the one observed in treatments employing IL-2 (198). In lymphopenic metastatic breast cancer patients during Phase II trial it increased CD4+ and other T-cell subset counts without altering their function (194, 195). Combining rIL-7 with vaccine therapy was used in two completed phase I clinical trials in patients with melanoma and pediatric solid tumors (182, 193). A phase II trial in patients with prostate cancer used glycosylated rIL-7 after vaccine therapy (185) (**Table 2**). The glycosylated version of rIL-7 will also be used in Phase II study in patients with locally advanced bladder urothelial carcinoma in a combination with Atezolizumab, an anti-PD-L1 antibody (208). The phase II study using chimeric antigen T cell therapy (CAR-T) in treating malignant solid tumor is planned to use patient's T cells and engineer them into IL-7 and Chemokine (C-C Motif) Ligand 19-expressing CD19-CAR-T cells and transfuse them into the patient for treatment of their B cell lymphoma (209).

A fusion protein, IL-7-Fc, composed of a recombinant form of IL-7 and a hybrid Fc region of a human antibody has been shown to stimulate proliferation and survival of different T-cell subsets and enhance anti-tumor immune responses (202). A phase I study on healthy volunteers was completed with further clinical trials at the moment either recruiting patients or planned for treatments of different types of malignancies: high risk skin cancer treatment in combination with Atezolizumab, treatment of high-grade glioma, treatment in combination with cyclophosphamide in patients with solid tumors being some of them (200, 210–212). Another phase I study will test the effects of IL-7-Fc on enhancement of immune reconstitution and vaccine responses in older people following chemotherapy due to their weakened immune system (213). Fc-fused IL-7 could also be used for inducing humoral immunity against viruses and a phase I clinical trial in human papilloma virus infected patients has been completed (201, 203, 214). Additionally, preclinical studies imply that IL-7-Fc can be used as an adjuvant in DNA vaccines and improve the immunogenicity (215, 216) (**Table 2**).

PERSPECTIVES AND CONCLUSIONS

The landscape of therapeutic agents that can modulate the bioactivity of TSLP and IL-7 in inflammation, autoimmunity and cancer is clearly very broad in terms of disease coverage and displays a strong focus on biologics. Indeed, a number of therapeutic agents have either already entered the market or are in the final stages of clinical studies as demonstrated (217, 218). The diversity of agents that have been developed to block TSLP action demonstrates the range of possibilities and approaches that could be used to alter the biological activity of cytokines in general. Although most of the reported signal mediators of TSLP seem to be developed with the intention of treating inflammatory allergic diseases with Tezepelumab being the most promising novel therapeutic for asthma treatment, these agents should also be considered in the future in other, non-allergen induced conditions such as leukemia or autoimmune diseases. Precise dissection of the role of TSLP in each type of solid cancers will be key to enabling appropriate therapeutic strategies. For instance, in cancer types where TSLP might be tumor-protective, recombinant TSLP could prove to have therapeutic value either independently, or in the form of an immunocytokine fusion for tumor suppression by analogy to IL-2 (219, 220). Leveraging on the available structural data on the TSLP-receptor complex together with diverse display techniques to select hits with tailored characteristics could be considered in the design of TSLP antagonists or agonists (115, 221, 222).

Targeting upstream signaling mechanisms by different therapeutic approaches is considered to be potentially beneficial in preventing relapse and maintaining remission in patients with chronic inflammatory disorders or autoimmune diseases (223).

For instance, this is supported by evidence that blocking the IL-7 mediated pathway can reverse ongoing autoimmunity (224). In the context of autoimmune diseases and cancer, the IL-7 signaling axis has been extensively targeted with antibodies against IL-7Rα which is rational considering that the effect of blocking γc chain could be problematic and lead to severe side effects since it is shared with numerous other cytokines (225). This approach has led to the identification of neutralizing antibodies that have completed phase I clinical trials and could potentially become beneficial for patients with autoimmune diseases. An additional reason for addressing IL-7Rα in drug development are frequent mutations leading to homodimerization of the receptor chains and constitutive signal transduction in a high percentage of B-ALL and T-ALL patients.

Considering the important role of both TSLP and IL-7 in the pathogenesis of RA simultaneous inhibition of both TSLP and IL-7R signaling in arthritis could serve a plausible therapeutic rationale in arthritis (226).

Although characterized by high selectivity, high efficacy, and limited side effects, biologics generally face a number of challenges such as expensive production, low tissue penetration and invasive administration (227–229). The design of small molecule inhibitors, an approach that highlights the importance of available structural information for facilitation of the design processes could present a suitable parallel alternative successfully addressing some of those issues. Ongoing and future studies on the diverse roles of TSLP and IL-7 in physiology and disease will undoubtedly further fuel efforts in the targeting of the two pleiotropic cytokines and their receptors in autoimmune diseases and cancer via appropriate molecular modulators.

AUTHOR CONTRIBUTIONS

IM and SS designed the scope and thematic coverage of the article. IM reviewed the current literature, wrote the main draft of manuscript, and generated the figures. SS reviewed the drafts, provided conceptual and textual input, and approved the final version of the manuscript. Both authors contributed to the article and approved the submitted version.

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IL-34 Actions on FOXP3$^+$ Tregs and CD14$^+$ Monocytes Control Human Graft Rejection

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Cytokines are major players regulating immune responses toward inflammatory and tolerogenic results. In organ and bone marrow transplantation, new reagents are needed to inhibit tissue destructive mechanisms and eventually induce immune tolerance without overall immunosuppression. IL-34 is a cytokine with no significant homology with any other cytokine but that acts preferentially through CSF-1R, as CSF-1 does, and through PTPζ and CD138. Although IL-34 and CSF-1 share actions, a detailed analysis of their effects on immune cells needs further research. We previously showed that both CD4$^+$ and CD8$^+$ FOXP3$^+$ Tregs suppress effector T cells through the production of IL-34, but not CSF-1, and that this action was mediated through antigen-presenting cells. We showed here by single-cell RNAseq and cytofluorimetry that different subsets of human monocytes expressed different levels of CSF-1R, CD138, and PTPζ and that both CD4$^+$ and CD8$^+$ FOXP3$^+$ Tregs expressed higher levels of CSF-1R than conventional T cells. The effects of IL-34 differed in the survival of these different subpopulations of monocytes and RNAseq analysis showed several genes differentially expressed between IL-34, CSF-1, M0, M1, and also M2 macrophages. Acute graft-vs.-host disease (aGVHD) in immunodeficient NSG mice injected with human PBMCs was decreased when treated with IL-34 in combination with an anti-CD45RC mAb that depleted conventional T cells. When IL-34-differentiated monocytes were used to expand Tregs *in vitro*, both CD4$^+$ and CD8$^+$ FOXP3$^+$ Tregs were highly enriched and this effect was superior to the one obtained with CSF-1. Human CD8$^+$ Tregs expanded *in vitro* with IL-34-differentiated allogeneic monocytes suppressed human immune responses in an NSG mouse aGVHD model humanized with hPBMCs. Overall, we showed that IL-34 induced the differentiation of human monocytes with a particular transcriptional profile and these cells favored the development of potent suppressor FOXP3$^+$ Tregs.

Keywords: IL-34, transplantation, tolerance, monocyte, Treg, cell therapy, GVHD, CSF-1R

INTRODUCTION

Organ and bone marrow transplantation is the only treatment for patients suffering from a number of diseases. In organ transplantation, the use of immunosuppressors has allowed remarkable success in the short and medium term graft survival, but unwanted side effects still lead to high morbidity and mortality, even when avoiding excessive immunosuppression (1). In bone marrow transplantation, acute and chronic GVHD are very frequent complications with high mortality and morbidity and thus with high unmet clinical needs (2, 3). In the long term, immunosuppressors can even be deleterious in the establishment of tolerance (4). Therefore, new treatments are needed that will be more specific for allogeneic immune responses and/or induce fewer side effects and that would allow, at the least, to decrease the use of immunosuppressors. Cytokines and enzymes controlling metabolic pathways have been described as powerful tools for controlling immune responses and it is important to identify new mediators of immune tolerance. Interleukin-34 (IL-34) is a cytokine, described for the first time in 2008 (5). Although IL-34 shares no homology with macrophage colony-stimulating factor (CSF-1 or M-CSF) in its amino acid sequence, they share a common receptor (CSF-1R or CD115) and IL-34 also has two distinct receptors, protein-tyrosine-phosphatase zeta (PTPζ) and CD138 (syndecan-1) (6, 7), suggesting additional roles for IL-34. In addition, the affinity of IL-34 for CSF-1R is higher than the one of CSF-1 and the binding mode to CSF-1R, as well as signaling of both cytokines, are different (8). Until now, studies have demonstrated that IL-34 is released by some cell types and is involved in the differentiation and survival of macrophages, monocytes, and dendritic cells (DCs) in response to inflammation, in the development of microglia and Langerhans cells (9, 10). More recent articles have described the immunoregulatory properties of IL-34 (11, 12). We have demonstrated that IL-34 is secreted by FOXP3$^+$ CD4$^+$ and CD8$^+$ regulatory T cells (Tregs) in human and CD8$^+$CD45RC$^{low/-}$ Tregs in rat. We also demonstrated that blockade of IL-34 *in vitro* in human and rat co-culture suppression assays inhibited both CD4$^+$ and CD8$^+$ Tregs suppressive function. Most importantly, we also showed that IL-34 treatment *in vivo* in a rat model of cardiac allograft induced transplant tolerance through the differentiation of macrophages toward a regulatory profile and subsequent induction of CD4$^+$ and CD8$^+$ Tregs by these macrophages (12). This role had never been evidenced before and needed to be explored in humans. We therefore investigated the tolerogenic effect of IL-34 on monocytes/macrophages and the mechanisms by which CD4$^+$ and CD8$^+$ Tregs were generated. Since CD4$^+$ and CD8$^+$ Tregs produce IL-34, our hypothesis was that IL-34 acts in autocrine and paracrine fashions to reinforce immune tolerance. Thus, we analyzed the expression of IL-34 receptors (CSF-1R, CD138, and PTPζ) on human monocytes and T cells and assessed the effect of IL-34 on human monocytes by single cell and bulk RNAseq. We also analyzed the effects of IL-34 on human Treg cell generation and evaluated in immune humanized mice the suppressive function of CD8$^+$ Tregs differentiated using IL-34-treated human monocytes in a model of acute GVHD.

In the present manuscript we report that IL-34 can act on CD14^{++}CSF-1R$^+$PTPζ$^+$ monocytes and CD4$^+$ or CD8$^+$ FOXP3$^+$CSF-1R$^+$ Tregs in an autocrine manner. We demonstrate that IL-34 action on monocytes results in differentiation toward a regulatory macrophage profile different from M2 macrophages, as shown by transcriptomic profiling. We demonstrate also that naive and effector precursor T cell depletion using anti-CD45RC mAbs results in synergistic enhanced IL-34 tolerogenic action *in vivo*. *In vitro*, we show that IL-34 is more efficient at inducing FOXP3$^+$ Tregs than CSF-1 and that these FOXP3$^+$ Tregs can efficiently control GVHD *in vivo* in a model of immune humanized immunodeficient mice.

Altogether, these data provide new informations on this new function of IL-34 on regulating Treg activity.

MATERIALS AND METHODS

Healthy Volunteers' Blood Collection and PBMC Separation

Blood from healthy individuals was obtained at the Etablissement Français du Sang (Nantes, France). Written informed consent was provided according to institutional guidelines. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque density-gradient centrifugation (Eurobio, Courtaboeuf, France). Red cells and platelets were eliminated using a hypotonic solution and centrifugation.

Cell Isolation

CD14^{++}CD16$^-$, CD14^{++}CD16$^+$, and CD14^{dim}CD16^{++} subsets were FACS Aria sorted from PBMCs based on size morphology and CD14$^{++/dim}$CD16$^{++/-}$ expression for differentiation with IL-34 (**Supplementary Figure 1E**). Total CD14$^+$ monocytes were isolated using a negative selection kit (Miltenyi Biotec., Bergisch Gladbach, Germany) for phosphorylation analysis, or by magnetic depletion (Dynabeads, Invitrogen) of CD3$^+$, CD16$^+$, and CD19$^+$, then FACS Aria sorting of CD14^{++} cells for both RNA sequencing analysis and Treg expansion. CD8$^+$ Tregs were obtained by enrichment of PBMCs in T cells (to 80% T cells) by magnetic depletion of CD19$^+$, CD14$^+$, and CD16$^+$ and then sorting of CD3$^+$CD4$^-$CD45RC$^{low/-}$ cells (**Supplementary Figure 4A**) using FACS ARIA II (BD Biosciences, Mountain View, CA, USA). Allogeneic APCs were isolated by magnetic depletion of CD3$^+$ cells from PBMCs.

Quantification of CSF-1R and PTPζ Signaling Pathway Activation

Freshly sorted CD14$^+$CD16$^-$ monocytes were plated at 1 $\times$ 10^6 cells/ml in fetal bovine serum (FBS)-free RPMI 1640 medium (1% penicillin-streptomycin, 1 mM glutamine, 1% NEAA, 10 mM Hepes, 1 mM sodium pyruvate) in low attachment round-bottomed 96-well plates (Perkin-Elmer, Inc., Waltham, MA, USA), and left untouched for 2 h before adding IL-34 or CSF-1 at a final concentration of 100 ng/ml. Analysis of the phosphorylation of AKT and ERK1/2 after 1, 3, 5, 10, and 15 min was performed by flow cytometry

following the BD Biosciences Phosflow protocol, using the BD Cytofix Fixation buffer and BD Phosflow Perm Buffer III (BD Biosciences), as well as phospho-AKT (Ser473) and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) primary goat antibodies (Cell Signaling Technology, Leiden, The Netherlands), and goat anti-rabbit IgG(H+L)-AF647 (Life Technologies, ThermoFisher Scientific) secondary antibody.

Differentiation of Monocytes and Expansion of Tregs

Monocytes were seeded at 1×10^6 cells/mL in complete RPMI 1640 medium supplemented with 10% FBS and IL-34 (2 nM, eBiosciences, ThermoFisher Scientific, Waltham, MA, USA) or CSF-1 (2 nM, R&D Systems, Bio-techne, Minneapolis, MN, USA) and macrophages were harvested at day 6. M1 macrophages were obtained by supplementing the medium with granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/mL, Cellgenix, Freiburg, Germany) over 5 days and by addition of interferon-gamma (IFNγ, 1000 U/mL, Miltenyi Biotec) from day 5 until day 7 of culture. M2 macrophages were obtained by supplementing the medium with CSF-1 (25 ng/mL, R&D Systems Biotechne) for 5 days and by addition of IL-4 (20 ng/mL, Cellgenix) and IL-10 (20 ng/mL, R&D Systems Biotechne) from day 5 until day 7 of culture. Lipopolysaccharide (LPS, 100 ng/mL, Sigma Aldrich, Saint-Louis, MO, USA) was added in the culture for the last 24 h for cytokine dosage. Macrophages were harvested using Trypsin (TryPLE, Gibco, ThermoFisher Scientific) at day 7.

Allogeneic PBMCs were seeded at 1×10^6 in 24-well plate in Iscove's modified Dulbecco's medium (IMDM), supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 5% human AB serum with IL-34- or CSF-1- differentiated macrophages at a ratio of PBMCs:macrophages 5:1 and cultured for 14 days.

$CD8^+CD45RC^{low/-}$ Tregs were seeded at 5×10^5 cells/cm²/500 µl in flat-bottom plates coated with anti-CD3 mAb (1 µg/mL, OKT3, hybridoma from the European Collection of Cell Culture), in complete RPMI 1640 medium supplemented with 10% FBS, IL-2 (1,000 U/mL, Proleukin, Novartis), IL-15 (10 ng/mL, Miltenyi Biotec) and soluble anti-CD28 mAb (1 µg/mL, CD28.2, hybridoma from the European Collection of Cell Culture) in the presence of IL-34-differentiated macrophages or allogeneic APCs irradiated (35 Gy) at 1:4 Treg:IL-34-macrophage or APC ratio. $CD8^+$ Tregs were stimulated again using anti-CD3 and anti-CD28 mAbs at day 7 of culture and IL-2 and IL-15 were freshly added at days 0, 2, 4, 7, 10 and 12.

Monoclonal Antibodies and Flow Cytometry

Antibodies used are listed in **Table 1** and **Supplementary Table 1**. For analysis of intracellular cytokines, Tregs were incubated with PMA, ionomycin, and brefeldine A (10 µg/ml) for 4 h before staining. Fc receptors were blocked (BD Biosciences) before staining and cells were permeabilized with a Fix/Perm kit (Ebiosciences).

TABLE 1 | List of antibodies used.

Marker	Clone	Provider
CD14	M5E2	BD Biosciences
CD16	3G8	BD Biosciences
CD115	9-4D2-1E4	BD Biosciences
PTPζ	Polyclonal	Bioss
CD138	MI15	BD Biosciences
CD3	SK7	BD Biosciences
CD4	RPA-T4	BD Biosciences
CD8	RPA-T8	BD Biosciences
CD25	M-A251	BD Biosciences
CD45RC	MT2	IQProduct
CD19	HIB19	BD Biosciences
CD56	B159	BD Biosciences
CD335	9E2/Nkp46	Biolegend
CD86	2331	BD Biosciences
CD80	L307.4	BD Biosciences
CD40	5C3	BD Biosciences
CD206	19.2	BD Biosciences
CD169	7-239	BD Biosciences
CD163	GHI/61	BD Biosciences
CD209a	DCN46	BD Biosciences
CD36	HIT2	BD Biosciences
CD1a	HI149	BD Biosciences
IL-34	578416	R&D System
TGFβ1	TW4-9E7	BD Biosciences
FOXP3	259D/C7	BD Biosciences
IFNγ	B27	BD Biosciences
Tbet	O4-46	BD Biosciences
GITR	REA841	Miltenyi Biotec
PD-1	EH12.1	BD Biosciences
CD127	hIL-7R-M21	BD Biosciences
CD28	CD28.2	BD Biosciences
CD27	M-T271	BD Biosciences
CD45RA	HI100	BD Biosciences
HLA-DR	L243	BD Biosciences
CD154	TRAP1	BD Biosciences
TRAIL	RIK-2	BD Biosciences
CD103	Ber-ACT8	BD Biosciences
hCD45	HI30	BD Biosciences
mCD45	30-F11	BD Biosciences
Phospho-Akt (Ser473)	D9E	Cell Signaling Technology
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	D13.14.4E	Cell Signaling Technology

Fluorescence was measured with LSR II or Canto II cytometers (BD Biosciences) and analyzed with FLOWJO software (Tree Star, Inc., Ashland, OR, USA).

ELISA

IL-10 and IL-12p40 were quantified in the supernatant of monocytes cultured for 6 days as well as control M1 macrophages, and both were stimulated for the last 24 h with LPS

at 100 ng/ml using Human IL-10 ELISA Set and Human IL-12p40 ELISA Set performed according to manufacturer's instructions (BD Biosciences).

DGE-RNA Sequencing

$CD14^{++}CD16^{-}$ monocytes were sorted by FACS Aria and lysed in RLT Buffer (Qiagen). RNeasy-Mini Kits (Qiagen) were used to isolate total RNA that was then processed for RNA sequencing. A protocol of 3′ Digital Gene Expression (DGE) RNA-sequencing was performed as previously described (13). Library was run on an Illumina NextSeq 550 high-output (2 × 75 pb) (Genom'IC platform, Cochin Institute, Paris). Reads 1 encode for well-specific barcodes and unique molecular identifiers (UMIs) whereas Reads 2 encode for 3' mRNA sequences and were aligned to human genome reference (hg19). Count matrix was generated by counting sample-specific UMI associated with genes for each sample. Differentially expressed genes between conditions were calculated using R package Deseq2 (Bioconductor) by first applying a regularized log transformation (rlog). Genes with adjusted *p*-value inferior to 0.05 were considered as differentially expressed. Heatmaps were generated by scaling and center genes expression. Finally, a volcano plot was designed by plotting -Log10 of adjusted *p*-value in function of log2 Fold Change; highlighted genes correspond to differentially expressed genes. The accession number for DGE-RNA sequencing raw data and processed data is GEO:GSE151194.

Single Cell RNAseq Analysis

An online public dataset of 10X genomics (https://support.10xgenomics.com/single-cell-gene-expression/datasets/3.0.2/5k_pbmc_v3_nextgem) was used to analyze gene expression of SDC1 (CD138), PTPRZ1 (PTPz) and CSF-1R in human PBMCs. Data were processed with "Seurat" package (version 3.1.3) in R software (RStudio, Inc., Boston). To eliminate unwanted cells (debris and doublets), cells with fewer than 200 genes or more than 4,000 genes were excluded. Then, cells with more than 10% of mitochondrial genes were excluded from the downstream analysis. Single cell transcriptomes were first normalized (log normalization) and then scaled. The most variable genes were found according to the variance stabilizing transformation (vst) method and were used to perform Principal Component Analysis (PCA). Clustering was performed on the first nine principal components, and hPBMC subsets were characterized according to expression of common membrane markers. Finally, a supervised analysis was performed to classify $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{dim}CD16^{++}$ monocytes.

Immune Humanized Mouse aGVHD Model

This study was carried out according to permit numbers APAFIS 3168 from the Ministry of Research. Eight to twelve-week-old NOD/SCID/$Il2r\gamma^{-/-}$ (NSG) mice were bred in our own animal facilities in SPF conditions (accreditation number C44-278). 1.5×10^7 human PBMCs were intravenously injected in 1.5 Gy-irradiated NSG mice the day before, as previously described (14, 15). Human PBMCs were monitored in blood and GVHD development was evaluated by body weight loss (14, 15). Human recombinant IL-34 (0.4 or 0.8 mg/kg/2.5 d for 20 days; from eBiosciences) and/or anti-human CD45RC mAbs (0.8 mg/kg/2.5 d for 20 days, MT2 or ABIS-45RC clones) were injected intraperitoneally. PBMCs were i.v. injected alone or with Tregs in a range of PBMC:Treg ratio from 1:0.5 to 1:2.

Statistical Analysis

Two-way repeated measure ANOVA was used to analyze mouse weight loss over time and Log Rank (Mantel Cox) test was used to analyze mouse survival. Friedman test with Dunn's multiple comparison test were used to compare monocyte frequency in PBMCs. Two-way ANOVA and Bonferroni post-test were used to analyze the survival of monocytes during the culture, phenotype of monocyte subsets and expanded Tregs. Mann Whitney *U*-test was used to compare the IL-10/IL-12p40 ratio in the supernatants of cultured macrophages.

RESULTS

CSF-1R and PTPζ Are Both Expressed on $CD14^{++}$ Monocytes and CSF-1R Is Also Expressed on $FOXP3^{+}$ $CD4^{+}$ and $CD8^{+}$ Tregs

We previously showed that IL-34 produced by $FOXP3^{+}$ Tregs acted at least on human monocytes *in vitro* (12). To get a better overview of IL-34 action on the immune system, we analyzed the expression of its reported receptors CSF-1R (also called CD115), CD138 (also called SDC1), and PTPζ (also called PTPRZ1) on whole PBMCs using a public single cell RNAseq dataset (https://support.10xgenomics.com/single-cell-gene-expression/datasets/3.0.2/5k_pbmc_v3_nextgem). We observed that CSF-1R single cell mRNA expression was restricted to monocytes and not significantly expressed by resting T, B and NK cells (**Figure 1A**). Analysis of markers of non-classical ($CD14^{dim}CD16^{++}$), intermediate ($CD14^{++}CD16^{+}$), or classical ($CD14^{++}CD16^{-}$) monocytes/macrophages (16, 17) showed that CSF-1R was expressed in all three populations of monocytes (**Figure 1B**) with a higher expression in non-classical and intermediate monocytes. In contrast, CD138 and PTPzeta mRNA expression was not detectable in resting PBMCs (**Supplementary Figures 1A,B**). However, we were able to detect PTPζ protein expression in all monocyte subsets and we also confirmed that CSF-1R was expressed by all monocytes, and both with a higher expression level in non-classical monocytes (**Figures 1C,D**). Nevertheless, since $CSF\text{-}1R^{+}$ and $PTP\zeta^{+}$ classical monocyte frequency in PBMCs is much higher than $CSF\text{-}1R^{+}$ and $PTP\zeta^{+}$ intermediate and non-classical monocytes (**Figure 1E**), it suggests that IL-34 will mostly act on $CD14^{++}CD16^{-}$ monocytes.

To better comprehend whether IL-34 could act directly on Tregs, we further analyzed CSF-1R and PTPζ expression in total $CD4^{+}$ or $CD8^{+}$ T cells compared to $FOXP3^{+}$ $CD4^{+}$ or $CD8^{+}$ Tregs (**Figure 1F** and **Supplementary Figures 1C,D**). We observed a significant expression of CSF-1R in non-stimulated $FOXP3^{+}$ $CD4^{+}$ and $CD8^{+}$ Tregs compared to total $CD4^{+}$ and $CD8^{+}$ T cells, respectively (**Figure 1F** and **Supplementary Figure 1C**). The expression was even higher

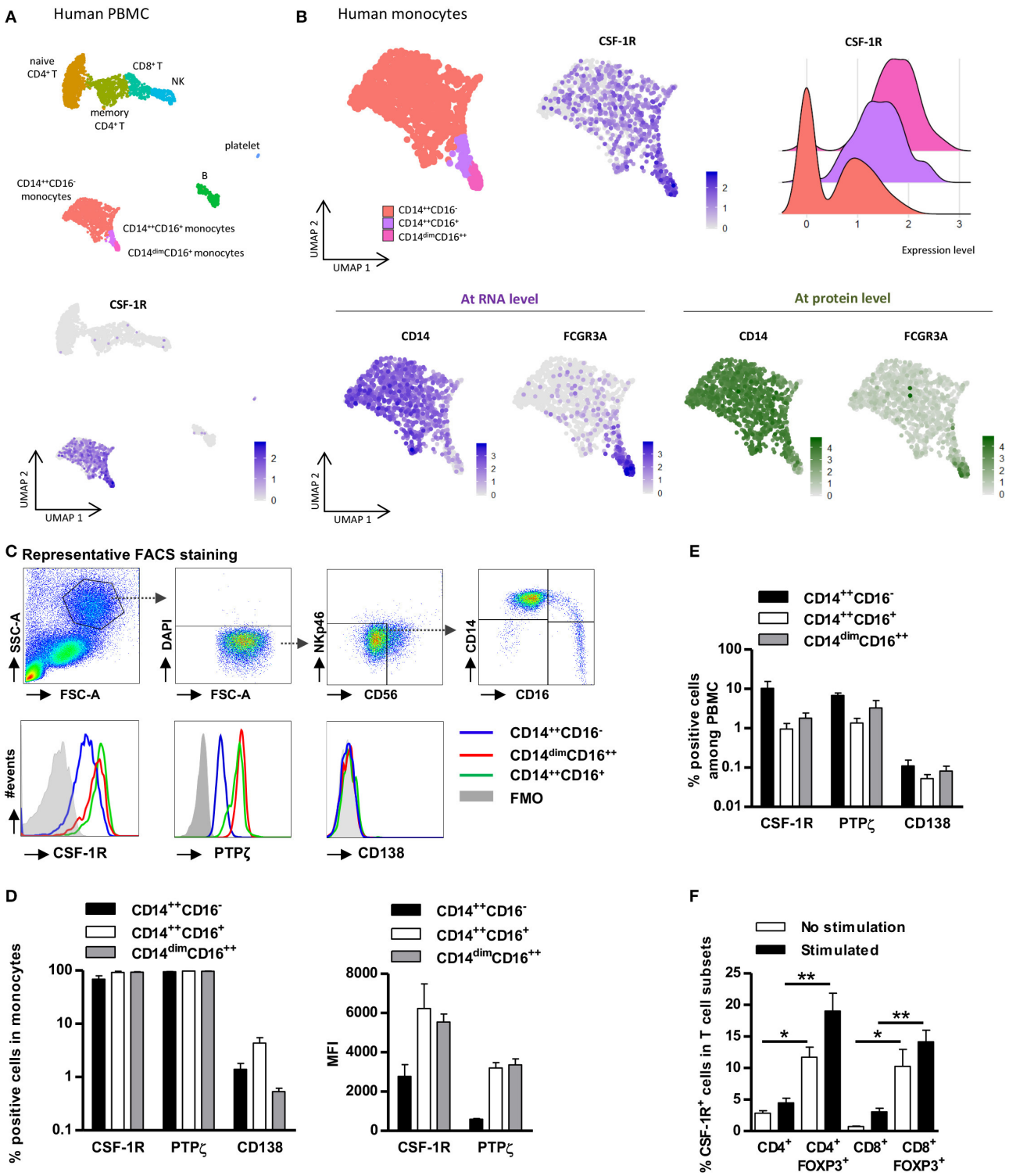

FIGURE 1 | CSF-1R and PTPζ expression is restricted to monocytes and FOXP3$^+$ Tregs. PBMCs were analyzed for CSF-1R expression at single cell transcriptional **(A,B)** and proteomic levels **(C–F)**. **(A)** Top: UMAP visualization of a public dataset of resting Human PBMC single cell RNA-seq from one healthy volunteer for which subsets of monocytes, T cells, B cells, and NK cells were identified by antibody staining. Bottom: CSF-1R expression in total PBMCs. One point represents one cell. Relative expression level is scaled from gray to dark blue. **(B)** Monocyte subsets were further subdivided based on RNA (RNAseq, bottom left) and protein expressions (CITEseq, bottom right) of CD14 (left) and FCGR3A (CD16) (right) summarized in the UMAP visualization (upper left), and subsets were analyzed for CSF-1R RNA expression (upper middle and right). One point represents one cell. Relative expression level is scaled from gray to dark blue (RNA expression) or from gray to dark

(Continued)

FIGURE 1 | green (protein expression). Upper Right: Violin plot representing the expression level of mRNA for CSF-1R in $CD14^{++}CD16^{-}$ monocytes (red), in $CD16^{++}CD14^{dim}$ monocytes (pink), and in $CD14^{++}CD16^{+}$ monocytes (purple). **(C)** Representative gating strategy for FACS analysis of CSF-1R, PTPζ, and CD138 expression in living ($DAPI^{-}$) non-NK cells ($CD56^{-}NKp46^{-}$) $CD14^{++/dim}CD16^{++/+/-}$ cell subsets from PBMCs. Representative from three individuals. **(D)** Frequency (left) of CSF-1R, PTPζ, and CD138 expressing cells and expression level (MFI) of CSF-1R and PTPζ (right) in $CD14^{++/dim}CD16^{++/+/-}$ cell subsets. $n = 3$ individuals. **(E)** Frequency of $CSF\text{-}1R^{+}$, $PTP\zeta^{+}$, and $CD138^{+}$ monocytes in total PBMCs. $n = 3$ individuals. **(F)** Frequency of CSF-1R expressing cells in stimulated (black) or not (white) $FOXP3^{+/-}$ $CD4^{+}$ or $CD8^{+}$ T cells. $n = 5$ individuals. Mann Whitney tests, $^{*}p < 0.05$, $^{**}p < 0.01$.

following stimulation, although it remains lower than on monocytes. We did not observe expression of PTPζ on Treg cells (**Supplementary Figure 1D**).

Altogether, these results suggest that IL-34 can act on $CD14^{++}$ monocytes, likely through CSF-1R and PTPζ and on $FOXP3^{+}$ Tregs through CSF-1R in PBMCs.

IL-34 Preferentially Acts Through $CD14^{++}CSF\text{-}1R^{+}PTP\zeta^{+}$ Monocytes to Induce Immunoregulation

We and others have shown that IL-34 induces differentiation of human $CD14^{++}$ monocytes into macrophages with regulatory properties (12, 18). However, we observed that CSF-1R and PTPζ expressions was higher on non-classical and intermediate than classical monocytes, thus we investigated in each of the three subpopulations the survival and maturation upon IL-34 treatment compared to M1- and M2-macrophages differentiated with GM-CSF+IFNγ or CSF-1+IL-4+IL-10, respectively, as controls (18, 19) (**Figure 2A** and cell sorting in **Supplementary Figure 1E**). Classical monocytes were largely predominant over intermediate and non-classical monocytes among PBMCs (about 18.8 vs. 4.7 vs. 1.8%, respectively, **Figure 2B**), and together with intermediate monocytes had a lower survival rate after 6-days culture than non-classical monocytes (10.6 vs. 24.7 vs. 21.2% for $CD14^{++}CD16^{-}$, $CD14^{dim}CD16^{++}$, and $CD14^{++}CD16^{+}$, respectively, **Figure 2C**). Comparing the phenotype, classical monocytes differentiated with IL-34 expressed higher levels than non-classical monocytes of M2-type markers CD163, CD36, CD169, CD206, CD14, and TRAIL (**Figure 2D** and **Supplementary Figure 1F**), displayed an anti-inflammatory cytokine secretion profile (**Figure 2E**), were isolated (vs. in clumps for non-classical differentiated monocytes) and displayed fewer dendrites under macroscopic observation (vs. intermediate and non-classical monocytes) (**Supplementary Figure 1G**). Intermediate monocytes had an intermediate phenotype, closer to classical than non-classical monocytes (**Figures 2D–E**). Interestingly, non-classical monocytes expressed high levels of the M2-associated marker CD209a after culture in the presence of IL-34 (**Figure 2D**). Finally, CD11b was more expressed in classical and intermediate monocytes, in accordance with previous observations (12, 20).

These results show that IL-34 is more efficient at inducing M2-like macrophages from classical and intermediate monocytes than non-classical monocytes and suggest that $CD14^{++}CSF\text{-}1R^{+}PTP\zeta^{+}$ monocytes are the cells through which IL-34 induces immunoregulation.

IL-34 Efficiently Induces Regulatory Macrophages From Classical Monocytes Expressing Different Genes Than CSF-1-Treated Macrophages

We further investigated the signal induced in $CD14^{++}CD16^{-}$ classical monocytes by IL-34 after binding the CSF-1R and PTPζ receptors in comparison to the signal induced by CSF-1 binding CSF-1R only. We observed a significant increase in the levels of phosphorylated AKT (**Figure 3A**) and ERK1/2 (**Figure 3B**) at 3 and 5 min following the addition of both IL-34 and CSF-1, compared to medium alone. CSF-1 induced non-significant slighter and higher levels of AKT and ERK1/2 phosphorylation compared to IL-34. After 6 days of culture, we observed morphological differences in the presence of IL-34 compared to CSF-1, with fewer dendrites and a more rounded morphology for IL-34-differentiated macrophages (**Figure 3C**), suggesting a difference in the phenotype of the differentiated macrophages. To further understand the similarities and differences of the IL-34 vs. CSF-1 induced macrophages, we performed a 3' digital gene expression RNA-sequencing (DGEseq) and compared freshly isolated $CD14^{++}$ monocytes (M0), 6-days differentiated macrophages in the presence of GM-CSF+IFNγ (M1), CSF-1+IL-4+IL-10 (M2), IL-34 alone, or CSF-1 alone (**Figures 3D–F**). Transcriptomic clustering (**Figure 3D**), principal component (**Supplementary Figure 2A**), and Pearson correlation (**Supplementary Figure 2B**) analyses highlighted the transcriptional changes following differentiation and indicated clear divergence between $CD14^{++}$ monocytes (M0) and M1-macrophages vs. all other groups and a clear convergence between M2-macrophages, IL-34-macrophages, and CSF-1-macrophages (**Figure 3D** and **Supplementary Figures 2A,B**). Further analysis of significant genes differentially expressed between IL-34 and CSF-1-macrophages revealed differential expression of 61 genes, with an upregulation of the expression of some interesting genes. Among those genes, we identified *PDK4,* a metabolic checkpoint for macrophage differentiation, *CHI3L1,* a carbohydrate-binding lectin that may play a role in tissue remodeling and cell capacity to respond to the environment involved in regulating Th2 cell responses and M2 macrophages differentiation, *FCER1A,* a receptor expressed by DCs that can play pro- or anti-inflammatory roles, and *CD300A,* a cell membrane receptor that contains classical ITIM motifs and negatively regulates Toll-like receptor (TLR) signaling mediated by MYD88 through the activation of PTPN6 and of macrophages in animal models (21). In contrast, we observed a down-regulation of *MARCO*, a marker of pro-inflammatory macrophages in IL-34-differentiated macrophages compared to CSF-1-differentiated macrophages

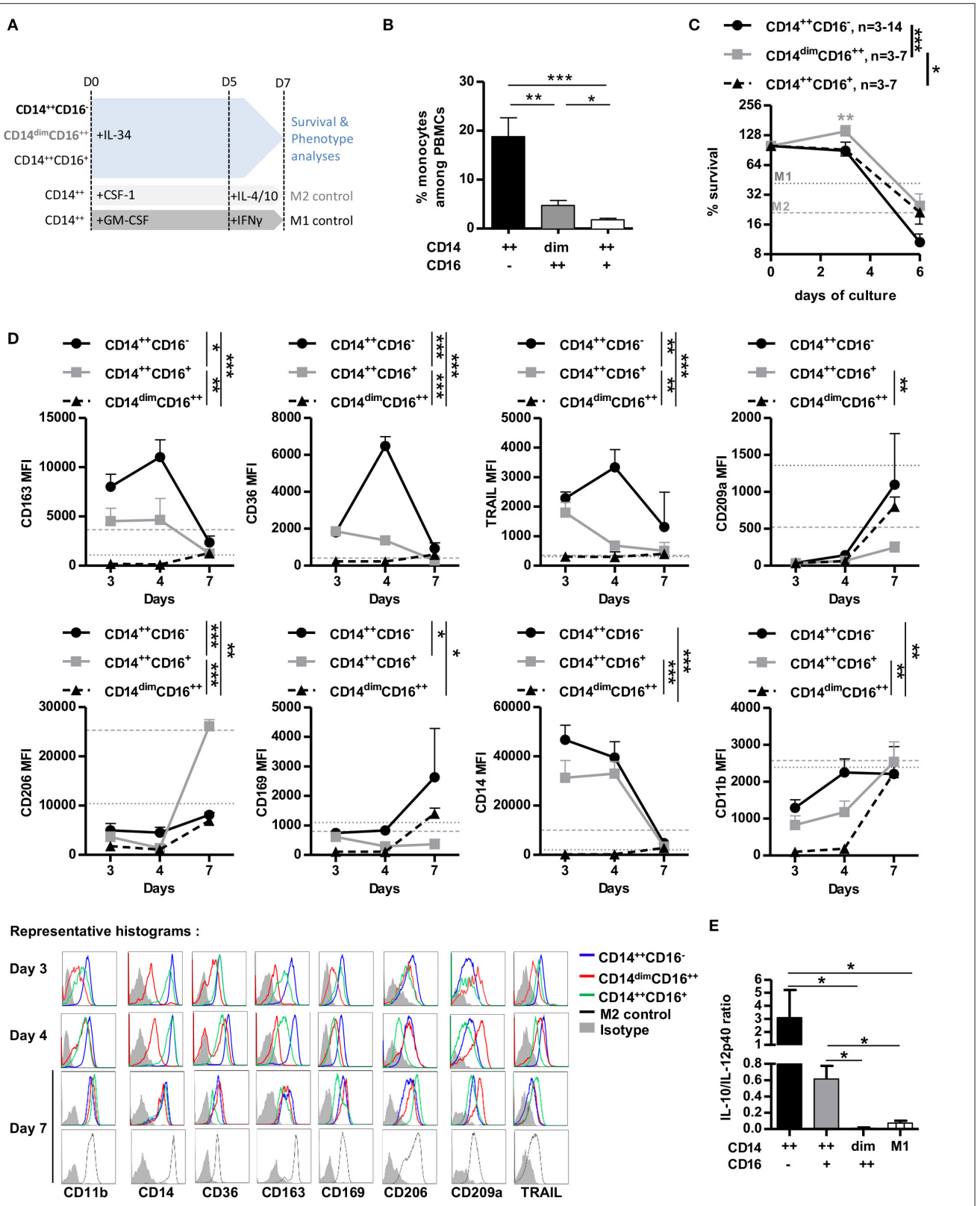

FIGURE 2 | CD14^{++} monocytes are the main mediators of IL-34-induced immunoregulation. **(A)** Schematic depicting conditions and timing of supplementation in cytokines in monocyte cultures. LPS was added for the last 24 h for cytokine release analysis only. **(B)** Frequency of monocyte subsets in PBMCs of healthy

(Continued)

FIGURE 2 | individuals. $n = 8$ individuals. Mann Whitney tests, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(C)** Living cell count over 6-days culture normalized to day 0 (=100%). $n = 3$–14 individuals. M1 (dark gray dotted line) and M2 (light gray dashed line) macrophages mean survival of three individuals after 7-days culture is shown. Two-way ANOVA and Bonferroni post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(D)** Monocyte subsets were cultured for 7 days in the presence of IL-34 and analyzed for surface marker expression. Top: Geometric mean of fluorescence +/– SEM out of three experiments is represented over time. M1 (dark gray dotted line) and M2 (light gray dashed line) macrophages mean of fluorescence of three individuals after 7-days culture is shown. Mann Whitney *U*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Bottom: Representative histograms of FACS staining. $CD14^{++}CD16^{-}$ (blue line), $CD14^{dim}CD16^{++}$ (red line), and $CD14^{++}CD16^{+}$ (green line). Isotypic control is shown in filled gray. **(E)** IL-10/IL-12p40 ratios secreted by LPS-activated macrophages were quantified in supernatants at day 6. $n = 3$–5 individuals. Mann Whitney *U*-test, $^{*}p < 0.05$.

(**Figure 3E** and **Supplementary Figure 2C**). Interestingly, further analysis of typical markers of macrophages (22) showed a preferential expression of some genes, such as *arginase-1* (*ARG1*) in IL-34 macrophages, compared to CSF-1, M1, and M2-differenciated macrophages, or *IDO1* that was found expressed only in M1 macrophages (**Figure 3F**). Other genes, like *IL-10,* in contrast were expressed by M2, IL-34, and CSF-1 macrophages.

Thus, IL-34 induced a high activation of monocytes through CSF-1R, subsequently inducing macrophages with a specific signature conferring regulatory/anti-inflammatory functions.

IL-34 Prolongs Survival in a Model of Humanized Acute GVHD Through Treg Expansion Rather Than Generation of Induced Treg From Naive T Cells

We highlighted previously that IL-34 treatment in a model of cardiac allo-transplantation resulted in the induction of highly suppressive Tregs through M2-like macrophages *in vivo* in rat and *ex vivo* in human (12). However, whether IL-34-induced Tregs resulted from the expansion of natural pre-existing Tregs or from newly converted Tregs from naive/effector T cells was not clear. Thus, we used an anti-CD45RC antibody (mAb) that specifically eliminates naive and precursor effector T cells (Teff) (13) and depleted *in vivo* $CD45RC^{high}$ Teff cells using a short-term course of anti-CD45RC mAb (as we previously described) in immunodeficient NOD/SCID/IL2rγ^{null} (NSG) mice injected with human PBMCs with or without IL-34 administration (**Figure 4A** and **Supplementary Figures 3A,B**). We observed that low-dose anti-CD45RC mAb treatment significantly delayed GVHD occurrence from 13.25 ± 0.9 days (mean survival) to 22.67 ± 2.7 days (**Figures 4B,C**). Although, low dose IL-34 treatment every 2.5 days at 0.8 mg/kg over 20 days was not sufficient to delay GVHD; IL-34 recombinant protein in combination with anti-CD45RC mAb therapy synergized and inhibited GVHD mortality in 66% of mice (**Figures 4B,C**). Analysis of mouse blood showed an efficient depletion of $CD45RC^{high}$ cells during the anti-CD45RC mAb treatment with no impact on the engraftment of other human PBMC subsets (**Supplementary Figure 3**).

These results suggest that Teff cell depletion in combination with IL-34 administration can more efficiently control immune responses.

IL-34 Induces, More Efficiently Than CSF-1, $FOXP3^{+}$ Tregs Which Delay Xenogeneic GVHD

We have previously shown that long-term tolerance in an allogeneic transplant model in rats treated with IL-34 was due to $CD4^{+}$ and $CD8^{+}$ Tregs that can control transplant rejection upon adoptive cell transfer (12). We also showed that human Tregs expanded from total PBMCs in the presence of IL-34-differentiated allogeneic macrophages suppressed immune response *in vitro* more potently than Tregs generated with monocytes in the absence of IL-34 (12). However, we did not assess whether this effect was comparable between IL-34 and CSF-1 or how these Tregs generated with IL-34 *in vitro* behaved *in vivo*. To do so, $CD14^{++}$ monocytes from healthy volunteers were cell-sorted and differentiated in the presence of IL-34 or CSF-1 for 6 days and then added to allogeneic PBMCs for 14 days in the presence of IL-2 and IL-15 and a polyclonal stimulation. We thus observed that in both $CD4^{+}$ and $CD8^{+}$ T cells, IL-34 increased more efficiently the frequency of $CD25^{+}FOXP3^{+}$ Tregs than CSF-1 (**Figures 5A,B**), and this increase was even more significant for $FOXP3^{+}CD8^{+}$ Tregs for which CSF-1 had little effect (**Figure 5B**). In addition, analysis of the number of $CD4^{+}$ and $CD8^{+}$ Tregs following a 14-day expansion in the presence of IL-34-differentiated macrophages demonstrated a higher number of total Tregs (both $CD4^{+}$ and $CD8^{+}$) compared to expansion in the presence of CSF-1-differentiated macrophages (**Figure 5C**).

We previously reported that polyclonal or chimeric antigen receptor (CAR)-modified $CD8^{+}$ Tregs can be efficiently expanded *in vitro* and control xenogeneic GVHD *in vivo* (14, 15). Given the efficacy of IL-34 to preferentially expand $FOXP3^{+}$ Tregs, we then assessed the therapeutic benefit of using IL-34 in the $CD8^{+}$ Treg expansion process for cell therapy. For this, we cultured naive $CD8^{+}CD45RC^{low/-}$ Tregs from PBMCs for 14 days in the presence of macrophages differentiated from $CD14^{++}$ monocytes by IL-34 compared to freshly isolated APCs, IL-2, and IL-15 cytokines, and a low polyclonal anti-CD3/anti-CD28 mAbs stimulation (**Figure 5D** and **Supplementary Figure 4A**). We obtained more than an 100-fold expansion of $CD8^{+}$ Tregs with either IL-34-differentiated macrophages (named IL-34-Tregs) or untreated macrophages (named Tregs) (**Figure 5E**). After expansion, IL-34-Tregs were highly enriched in $FOXP3^{+}$ cells, expressed higher levels of surface markers commonly related to $CD4^{+}$ and $CD8^{+}$ Tregs, such as GITR and PD-1, and cytokines such as TGFβ, IFNγ, and IL-34 that we have demonstrated as being

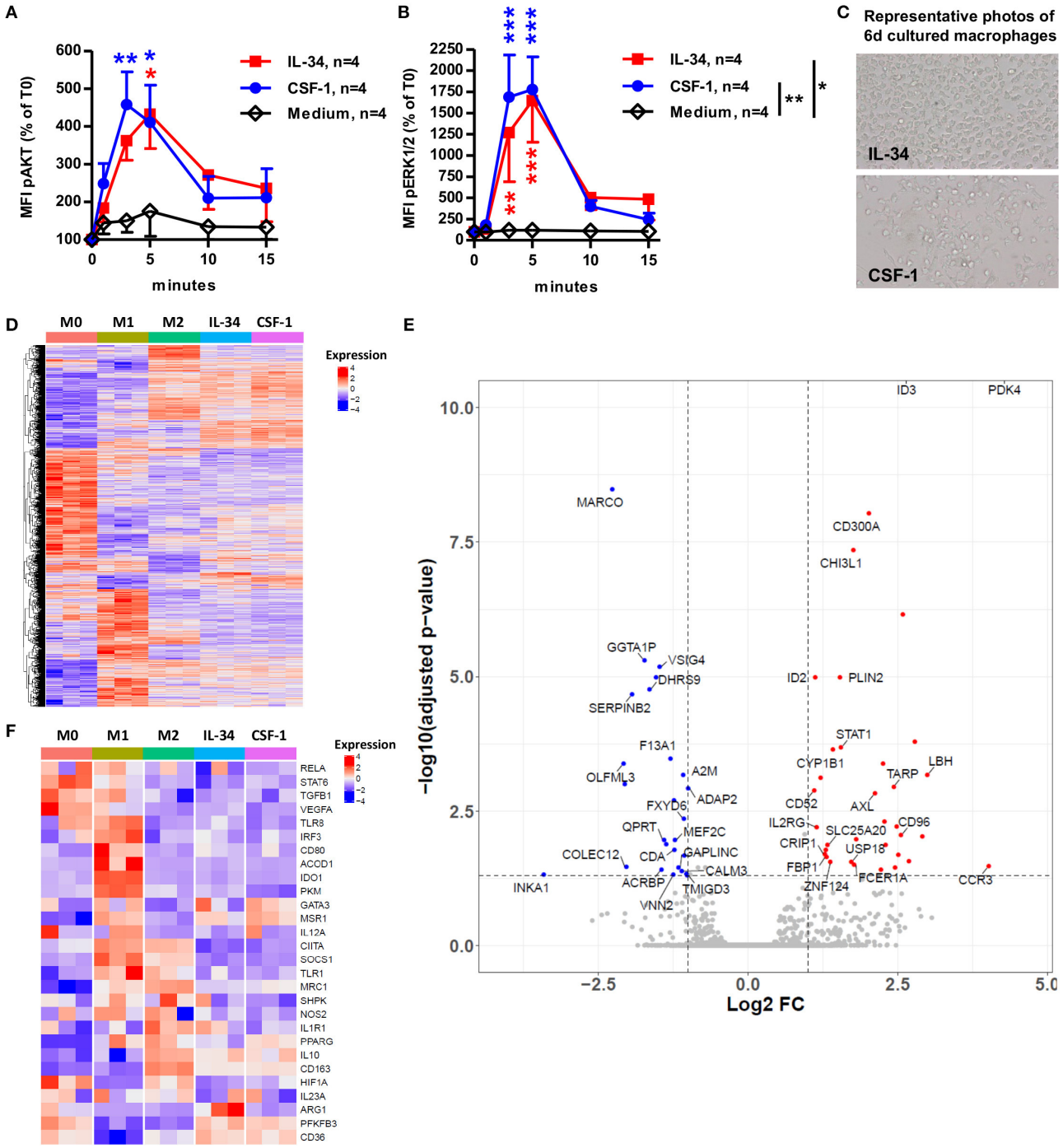

FIGURE 3 | IL-34-induced macrophages display a transcriptome close, but not identical, to M2-type and CSF-1-induced macrophages. **(A,B)** CD14^{++} monocytes were cultured with IL-34 or CSF-1 for 1, 3, 5, 10, and 15 min and analyzed for phosphorylation of AKT **(A)** and ERK1/2 **(B)** by flow cytometry. Results are represented as a percentage of baseline levels (T0). $n = 4$ individuals. Two-way ANOVA and Bonferroni post-test compared to medium alone. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(C)** Photos of CD14^{++} monocytes after 6 days of culture in the presence of IL-34 or CSF-1. X20 magnification. **(D–F)** CD14^{++} monocytes were cultured for 6 days with IL-34 or CSF-1 and analyzed by DGE-RNAseq for gene expression. **(D)** Expression levels of differentially expressed genes between each condition are presented as a heatmap. Each column represents one sample. Blue color represents low expressed genes and red color represents highly expressed genes. The color bar shows experimental conditions. M0 are freshly sorted monocytes. **(E)** Volcano plot highlighting overexpressed genes (on the right, red dots) and under-expressed genes (on the left, blue dots) in IL-34-differentiated macrophages as compared with CSF-1-differentiated macrophages. The *p*-value adjusted cut-off is 0.05. **(F)** Heatmap representing expression of M1 and M2 macrophage genes in samples. Gene expression was normalized with regularized log transformations (rlog) algorithm (Deseq2), center and scaled. Blue color represents low expressed genes and red color represents highly expressed genes. Supervised clustering was performed to order samples. The color bar corresponds to experimental conditions.

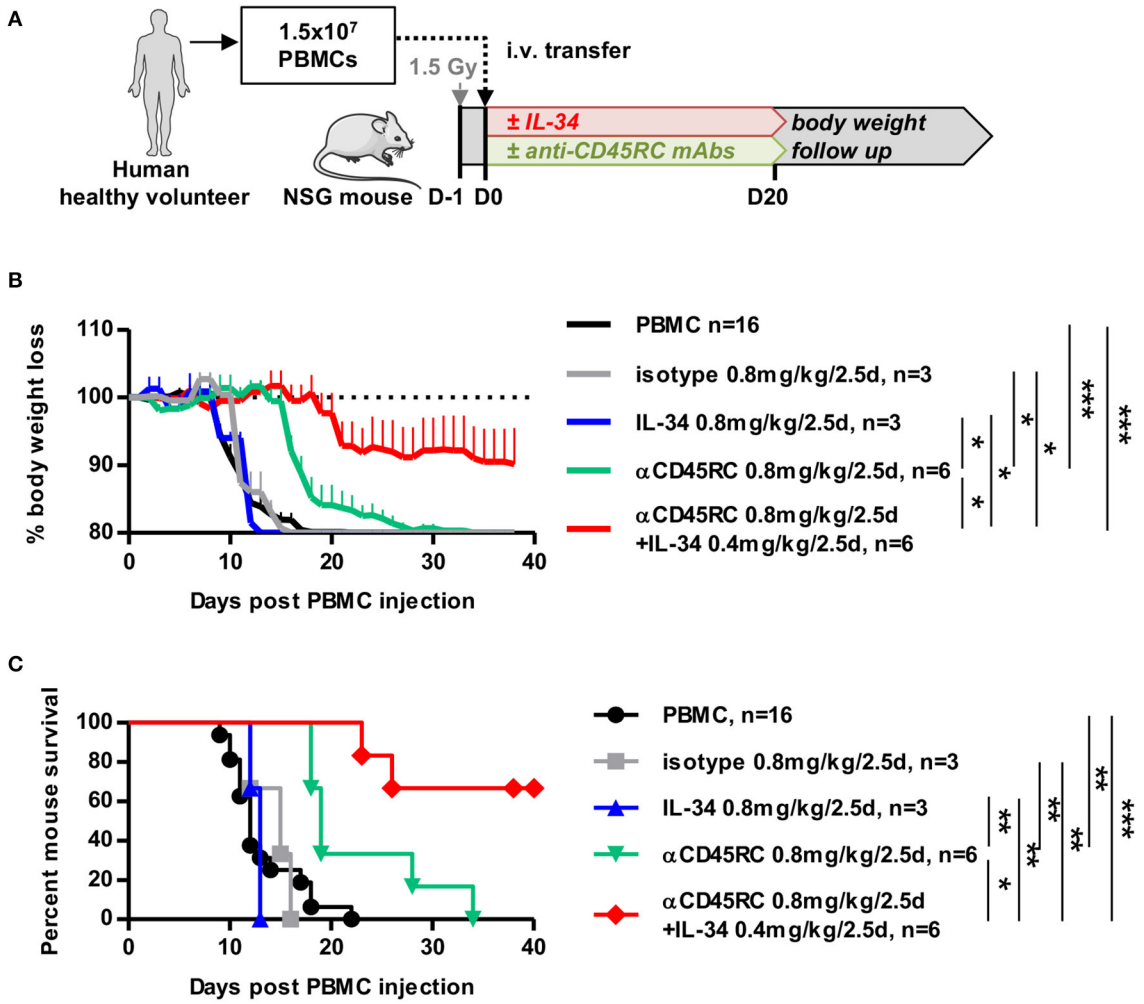

FIGURE 4 | IL-34 in combination with depletion of naive cells prolongs survival in an acute GVHD humanized model. **(A)** Schematic depicting the GVHD model in humanized mice. NSG mice were injected with human PBMCs, treated or not with IL-34 protein and/or anti-CD45RC mAbs for 20 days, and followed for body weight loss. **(B)** Evolution of mouse body weight over time, normalized to the weight before the injection of PBMCs (D0), after no treatment (black line), IL-34 treatment (blue line), anti-CD45RC mAb treatment (green line), isotype Ig control treatment (gray line), and dual IL-34 + anti-CD45RC mAb treatment (red line). $n = 3$–16. Mean ± SEM is represented. Two Way repeated measure ANOVA, $^{*}p < 0.05$, $^{***}p < 0.001$. **(C)** Percentage of mouse survival over time. $n = 3$–16. Log Rank (Mantel-Cox) test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

mediators of $CD8^+$ Treg-suppressive activity (23, 24) (**Figure 5F** and **Supplementary Figure 4B**).

Finally, we assessed the suppressive function of IL-34-Tregs *in vivo* in a xenogeneic model of acute GVHD (**Figures 6A–C**). NSG mice were first injected with human PBMCs to induce a xenogeneic acute GVHD and were either treated or not with IL-34-Tregs in a range of PBMC:Treg ratios (**Figures 6A–C** and **Supplementary Figures 4C,D**). We observed that IL-34-Tregs significantly delayed body weight loss (**Figure 6B**) and mouse survival (**Figure 6C**) in a dose-dependent manner compared to the control group.

Altogether, these results demonstrate that IL-34 is beneficial for $FOXP3^+$ Treg expansion *ex vivo* and that $CD8^+$ Tregs expanded with IL-34 can control graft rejection in a dose-dependent manner.

DISCUSSION

Altogether, we have demonstrated that IL-34-treated $CD14^{++}CSF\text{-}1R^+PTP\zeta^+$ monocytes were differentiated into pro-tolerogenic macrophages with a specific signature able to efficiently expand and potentiate $FOXP3^+$ Tregs *in vitro* and *in vivo* to control anti-donor immune responses (**Figure 7**).

We found the expression of CSF-1R and PTPζ mostly on $CD14^{++}$ classical and intermediate monocytes, although we found a more significant expression of both receptors on non-classical $CD16^{++}$ monocytes (16, 25, 26). As for CSF-1, IL-34 could polarize all three subtypes of monocytes into type 2 (M2) macrophages depending on the environment (27). Non-classical macrophages in particular play an important role in the control of immune responses and have also been associated

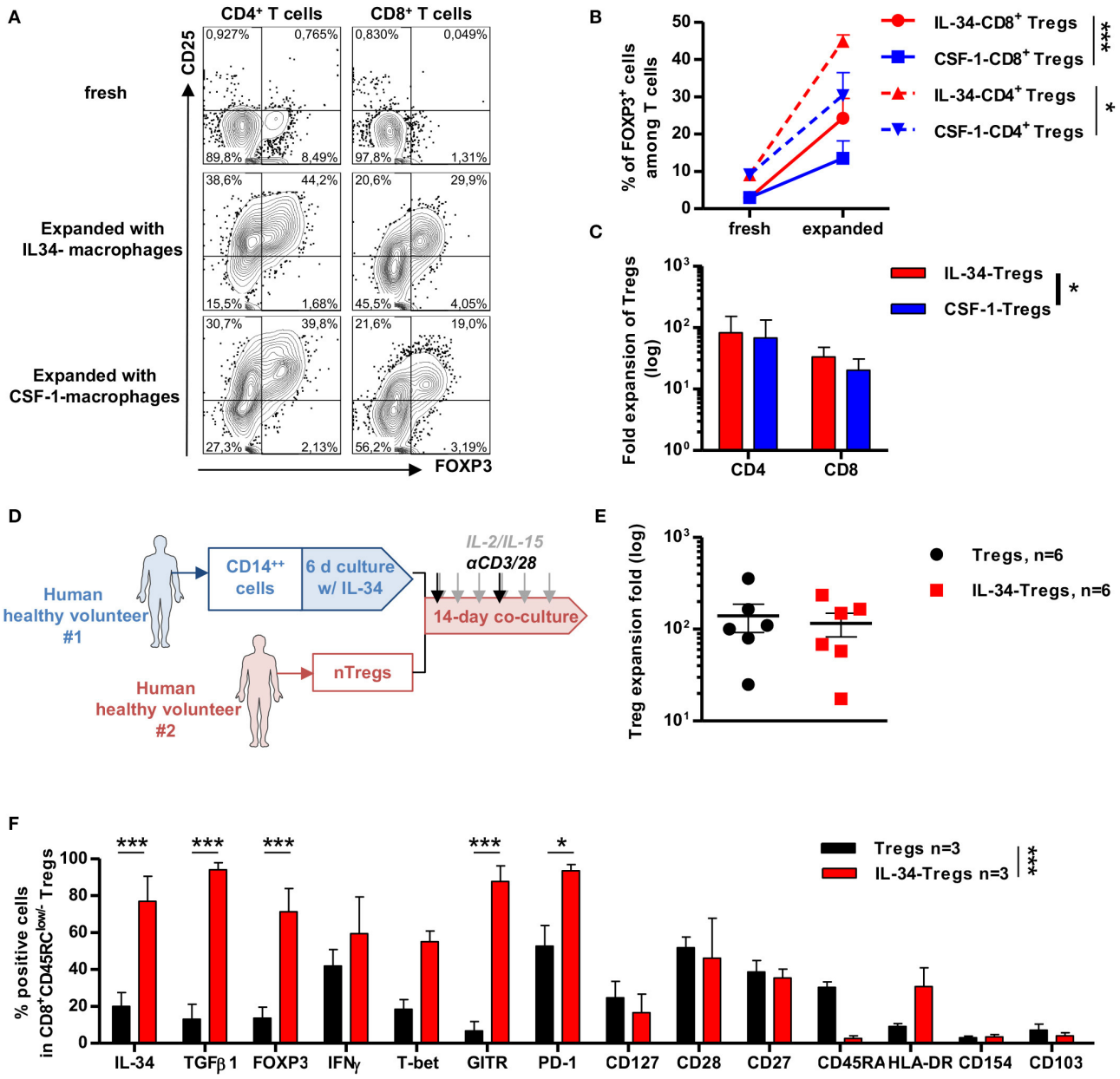

FIGURE 5 | IL-34 potentiates differentiation of CD4$^+$ and CD8$^+$ FOXP3$^+$ *in vitro* more effectively than CSF-1. **(A)** Representative FACS staining of CD25 and FOXP3 expression in CD4$^+$ (left) and CD8$^+$ (right) T cells after 14-day culture of PBMCs with either IL-34-differentiated macrophages (middle) or CSF-1-differentiated (bottom) macrophages compared to fresh cells (upper). **(B)** Frequency of FOXP3 positive cells in CD4$^+$ (dotted lines) and CD8$^+$ (solid line) T cells before and after expansion with IL-34- (red lines) or CSF-1- (blue lines) differentiated macrophages. Two-way ANOVA, $^*p < 0.05$, $^{***}p < 0.001$. **(C)** CD4$^+$ and CD8$^+$ Tregs count harvested after 14 days of culture with IL-34- (red bars) or CSF-1- (blue bars) differentiated macrophages in fold expansion. Two-way ANOVA, $^*p < 0.05$. **(D)** Schematic depicting cell culture. CD14^{++} monocytes were sorted from a healthy volunteer (HV#1), cultured for 6 days in the presence of IL-34, then added to CD8$^+$CD45RC$^{low/-}$ Tregs harvested from another healthy volunteer (HV#2) and 14-day cultured in the presence of a polyclonal stimulation once per week and IL-2 + IL-15 supplementation three times per week. **(E)** Treg cell count harvested after 14 days of culture with IL-34-differentiated macrophages or freshly isolated APCs normalized to Treg cell count seeded at day 0. **(F)** IL-34-Tregs (red bars) were analyzed by flow cytometry for Treg-associated marker expression as compared to before expansion (fresh cells, black bars). $n = 3$ individuals. Two-way ANOVA and Bonferroni post-test, $^*p < 0.05$, $^{***}p < 0.001$.

with wound-healing and resolution of inflammation in damaged tissues (28). PTPζ expression was mostly reported in the brain and, more recently, in the kidney (11, 29), while its expression on monocytes has only been suggested by western blotting (30); thus, our study confirms that both CSF-1R and PTPζ are expressed at the protein level by monocytes, suggesting that IL-34 action on monocytes through both PTPζ and CSF-1R could explain the differential effect compared to CSF-1. The intracellular signaling

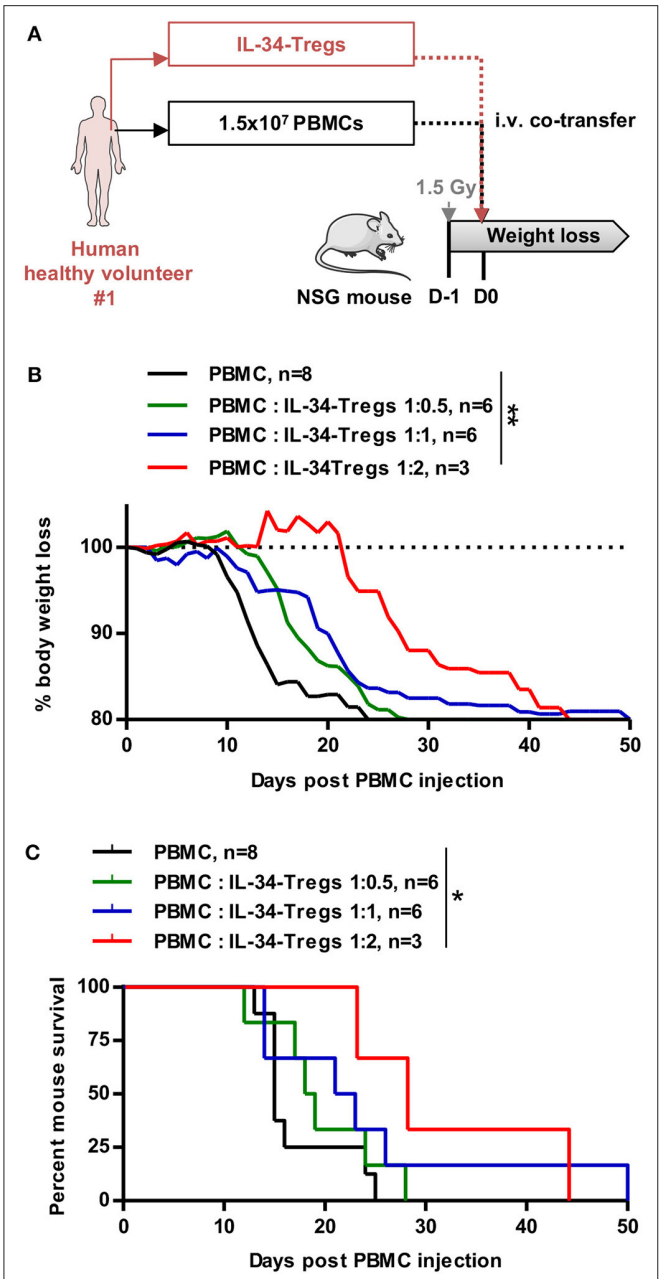

FIGURE 6 | Cell therapy with IL-34-expanded CD8$^+$ Tregs delays aGVHD. **(A)** Schematic depicting treatment of mice in the model of xenogeneic GVHD. PBMCs injected are syngeneic to the expanded Tregs co-injected. **(B)** Mouse body weight follow-up and **(C)** mouse survival after PBMC injection (D0) with or without Tregs expanded in the presence of IL-34-differentiated macrophages in a range of PBMC:Tregs ratio. $n = 3$–8. **(B)** Two-way RM ANOVA, $^{**}p < 0.01$. **(C)** Log Rank (Mantel Cox) test. $^{*}p < 0.05$.

through PTPζ in monocytes still needs to be analyzed. The differential effects of IL-34 and CSF-1 can also be explained by the different binding characteristics and signaling through the CSF-1R that are discussed below.

We did not observe CSF-1R and PTPζ expression on resting total T cells, including Tregs, in single cell RNAseq data analysis of total PBMCs, probably because of the low frequency of Tregs and the low frequency of CSF-1R in Tregs compared to monocytes. However, using antibody staining, we were able to find a low expression of the protein CSF-1R on resting CD4$^+$ and CD8$^+$ FOXP3$^+$ Tregs and upon stimulation this expression was significantly increased on activated CD4$^+$ and CD8$^+$ FOXP3$^+$ Tregs. Thus it is possible that IL-34 acts directly on Treg polarization as TGFβ and IL-2, or on Treg function, in addition to acting through monocytes (31), and this will need to be further investigated.

Surprisingly, we did not observe any expression of CSF-1R in expanded FOXP3$^+$ Tregs (data not shown), suggesting a transient expression of CSF-1R in Tregs upon activation and a narrow window for IL-34 to act directly on those cells. This further suggests a synergistic effect of IL-34 on monocytes and recently activated Tregs that supports the therapeutic strategy based on a short course treatment with IL-34 to induce tolerogenic monocytes and Tregs right after an immune challenge.

Although IL-34 and CSF-1 bind to the same receptor, CSF-1R, on the same cells, IL-34 can also act through PTPζ binding on monocytes, resulting in a different potential to induce FOXP3$^+$ Tregs *in vitro*. They are several hypotheses to explain this important difference in their respective capacity to induce FOXP3$^+$ Tregs (both CD4$^+$ and CD8$^+$). IL-34 and CSF-1 have very different sequences and structures, as well as a different affinity for CSF-1R (IL-34 has an affinity 34-fold superior to the one of CSF-1 for CSF-1R) (11, 32), and although they establish structurally similar binding to CSF-1R, it is possible that the subsequent signaling and the signaling and transcriptional pathways involved in the differentiation of the monocytes to macrophages and the phenotype of the differentiated macrophages are different (33, 34). The higher affinity of IL-34 to CSF-1R would suggest a more important signal transduction for IL-34 compared to CSF-1. In addition, the expression of PTPζ probably impacts on CSF-1R-signaling in monocytes. Whether PTPζ reinforces, weakens, fastens, or slows down the signal induced through CSF-1R needs further investigation. We observed that IL-34 and CSF-1 induced in a similar manner the phosphorylation of AKT and ERK1/2, two molecules involved in the signaling of both CSF-1R and PTPζ molecules. In addition, although we did not observe striking differences in the global transcriptomic profile of 6-days differentiated macrophages with either IL-34 or CSF-1, we did observe several functionally important genes differentially regulated. *Arginase-1* mRNA was highly and specifically increased in IL-34-differentiated macrophages. Arginase-1 degrades arginine, deprives NO synthase of its substrate, down-regulates nitric oxide production, and is one of the key factors by which regulatory macrophages or myeloid-derived suppressor cells suppress T cell responses (35, 36). Arginase-1$^+$ macrophages also promote wound-healing and decrease T cell activation and induce it when tolerance is sought or when targeting Arginase-1 in cancer is the focus of current efforts (37, 38). We also observed significant upregulation of other genes, such as *PDK4*, a metabolic checkpoint for macrophage differentiation (39), *CHI3L1*, a marker of M2

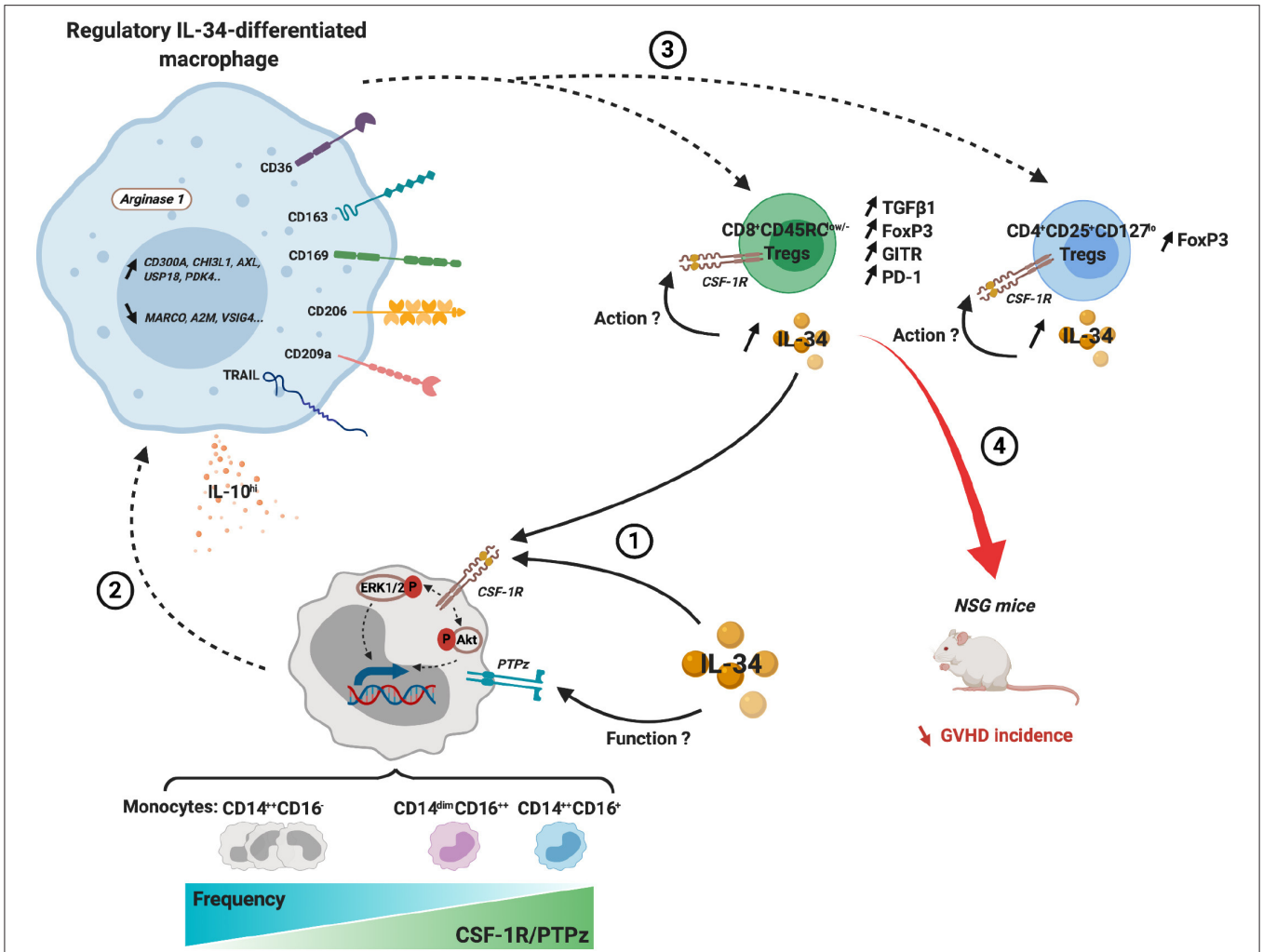

FIGURE 7 | Integrated scheme of the regulatory actions of IL-34-differentiated macrophages and their ability to potentiate FOXP3$^+$ Tregs. (1) IL-34 exogenously administered or from endogenous sources, such as from Treg, acts through CSF-1R to preferentially differentiate classical and intermediate monocytes into regulatory macrophages (2). (3) IL-34-differentiated macrophages expand and enhance the suppressive phenotype of both CD4$^+$CD25$^+$CD127^{low} and CD8$^+$CD45RC$^{low/-}$ Tregs. IL-34 secretion by Tregs maintains and increases the regulatory loop and can act in an autocrine fashion on Tregs. (4) In GVHD in NSG mouse, expanded CD8$^+$ Tregs efficiently delay GVHD incidence. Dashed arrow, induction; solid arrow, binding.

macrophages (40), *FCER1A*, a receptor expressed by DCs and a few monocytes that can play pro- or anti-inflammatory roles (41, 42), or *CD300A*, a negative regulator of TLR signaling in IL-34-differentiated macrophages compared to CSF-1-differentiated macrophages, emphasizing the differences between IL-34 vs. CSF-1. Interestingly, we found several genes involved in macrophage phagocytosis downregulated [i.e., *MARCO* (43–45), *A2M* (46, 47), *VSIG4* (48), or *COLEC12* (49, 50)] or inhibitors of phagocytosis upregulated such as *CD300A* (51) in IL-34-differentiated macrophages compared to CSF-1-differentiated macrophages, suggesting a decreased capacity to phagocytes compared to CSF-1 (34), but this will need further investigation. Although we found a low number of genes differentially regulated between CSF-1- and IL-34-differentiated macrophages, these markers emphasized the difference of activity on CSF-1R and/or the impact of the exclusive binding of IL-34 on PTPζ. The role of these different genes on the observed promoting effect of IL-34 on Treg induction will also need further investigation.

The capacity of IL-34 to induce both CD4$^+$ and CD8$^+$ Tregs is interesting as it would suggest that both CD4$^+$ and CD8$^+$ FOXP3$^+$ cells could be expanded together without cell sorting from total PBMCs and then the final product, enriched in both Treg subsets, could be administered subsequently *in vivo*. Maybe elimination of Teff and naive cells using anti-CD45RC mAbs, for example, as we showed *in vivo* that it was beneficial for IL-34-therapeutic potential, would also be beneficial *in vitro* in the expansion protocol (i.e., depletion of CD45RC$^+$ cells by cell sorting). These results obtained with the anti-CD45RC mAb suggest that naive/effector T cells were not involved in IL-34 establishment of a control of immune responses and that Tregs were rather expanded cells than newly-generated cells. Although we cannot conclude on a direct effect of IL-34 on Tregs in this

experiment, since human IL-34 does not cross-react on murine cells and can only act on human cells and since in this model of humanized mice, GVHD is mediated mostly by T cells, this suggests a direct effect of IL-34 on Tregs and will need to be the subject of further investigations. The synergy between IL-34 and anti-CD45RC mAb also suggests that *in vivo* IL-34 efficacy may be limited by Teff cells. Although the synergistic capacity of $CD4^+$ and $CD8^+$ Tregs is not yet clear, both subsets could show complementary effects and it could be beneficial to administer them together to patients (24). IL-34 could also be used *in vivo* together with Treg cell therapy to promote the persistence and the function of the induced Tregs, as is done with low-dose IL-2 or rapamycin (52, 53), by enrichment of the environment with tolerogenic macrophages and by direct action on Tregs. We have tested *in vivo* the $FOXP3^+CD8^+$ Tregs induced in the 14-day *ex vivo* expansion in a model of xenogeneic GVHD in immune-humanized mice, and we have observed a similar protective potential of the Tregs compared to what we have previously demonstrated using polyclonally expanded $CD8^+$ Tregs (14). Thus, it suggests that efficient Tregs were expanded, even from total PBMCs as a starting material, which shows similar protection compared to Tregs expanded without IL-34. Thus, an important advantage of using IL-34 would be the co-expansion of $CD4^+$ and $CD8^+$ $FOXP3^+$ Tregs from total PBMCs. Also, this suggests that upon improvement of this protocol, with for example selective effector T cell depletion before expansion, it could result in improved protection.

Altogether, our results highlight the potential of IL-34 to favor the development of $FOXP3^+$ Tregs and suggest that this cytokine should be further considered for *in vitro* use or *in vivo* therapy.

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 Ministry of Research. Blood from healthy individuals was obtained at the Etablissement Français du Sang (Nantes, France). Written informed consent was provided according to institutional guidelines.

AUTHOR CONTRIBUTIONS

CG and IA contributed conception and design of the study. CG, SB, and IA wrote sections of the manuscript. SB, AF, CS, AS, and NV performed experiments and analyzed data. All authors contributed to manuscript revision, read, 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.2020.01496/full#supplementary-material

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Conflict of Interest: CG, IA, and SB have patents on IL-34 that are pending and are entitled to a share in net income generated from the licensing of these patent rights for commercial development.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cluster Analysis of Dry Eye Disease Models Based on Immune Cell Parameters – New Insight Into Therapeutic Perspective

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Dry eye disease (DED) can be represented as a display of disease in the mucosal part of the eye. It is quite distinct from the retinal side of the eye which connects with the neurons and thus represents the neuroimmunological disease. DED can occur either by the internal damage of the T cells inside the body or by microbial infections. Here we summarize the most common animal model systems used for DED relating to immune factors. We aimed to identify the most important immune cell/cytokine among the animal models of the disease. We also show the essential immune factors which are being tested for DED treatment. In our results, both the mechanism and the treatment of its animal models indicate the involvement of Th1 cells and the pro-inflammatory cytokine (IL-1β and TNF-α) related to the Th1-cells. The study is intended to increase the knowledge of the animal models in the field of the ocular surface along with the opening of a dimension of thoughts while designing a new animal model or treatment paradigm for ocular surface inflammatory disorders.

Keywords: dry eye disease, animal models, helper T cells, inflammation, therapy

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INTRODUCTION

Dry eye disease (DED) is characterized by the inflammation of the ocular surface and it involves the structures present in the mucosal portion of the eye like conjunctiva, cornea, meibomian glands, goblet cells, and lacrimal glands. In humans, the disease is associated with painful itchy eyes followed by chronic progressive phenotype leading to reduced vision and blindness. The history of DED can be traced back to the early 1900s when a Danish physician depicted the importance of two subclasses; the primary Sjögren's syndrome (pSS, related to dry eye or mouth) or the secondary Sjögren's syndrome (sSS, related to autoimmune diseases like rheumatoid arthritis) (1). DED being a multifactorial disease in the tear film and ocular surface suggests a dysregulation of the immune mechanism and leads to a cycle of continued inflammation in its chronic form (2). Though the disease is quite clearly phenotyped in human subjects, the exact pathogenesis and underlying immune mechanisms have only recently been understood with the help of DED animal models. The animal models used for DED research are rodent (mice, rats), canine (dogs), porcine (pigs), feline (cats), other mammals (sheep, rabbits), and also non-human primate models (3, 4).

The choice of animal model is very much dependent on the question researchers are investigating. For example, mice are mostly used for genetic manipulation, rabbits for the pharmacological experiments and toxicological testing, feline, canine, and porcine models are used for the determination of pathological features similar to humans, like the blinking rate in Schirmer's test. One of the most commonly used mice models is the NOD (non-obese diabetic) mice.

The environment plays a crucial role in DED because the ambient dehydrating environment can lead to changes in the disease phenotype and progression. That is why there are several DED animal models generated by modulating the ambient environment using, for example, a controlled-environment chamber or desiccating stress or scopolamine-induced models. Oxidative damage is induced in these animals with a change in the ambient environment like many other ocular disorders (e.g., cataract, acute macular degeneration). Mechanistically, the reactive oxygen species in these environment-induced dry eye models involve NLRP3 inflammasome activation and increasing IL-1β secretion through the activation of Caspase-1 (5). We have used both genetically modified and environment-induced DED models for our study, which are enlisted in the section "Materials and Methods."

The involvement of both innate and adaptive immune system pathways in DED is well documented (6, 7). Several cytokine molecules are identified to regulate or to trigger these pathways. For example, topical treatment with IL-1R-antagonist in C57BL/6 mice ameliorates disease whereas inhibition of TNF-α in the salivary gland of NOD mice can have a negative effect on salivary gland function as shown in (8, 9). This makes this disease even more complicated to treat through a common pathway/molecule/immune factor.

We aim to find out the most commonly used animal models in DED followed by the search for the most important immune cell contributing to disease progression. In our result, a cluster analysis of different cell types, cytokine signatures and different animal models depict the importance of both $CD4^+$ and $CD8^+$ Th cells, Th1 cytokines IFN-γ and TNF-α and IFN-γ-related cytokine CXCL9. Along with the mechanism of DED, we have evaluated the interest of this research area after the analysis of research papers from all over the world. Finally, we showed the most important cytokine molecule (IL-6, IFN-γ, TNF-α, and IL-1β) used for the treatment of DED animal models. It is clear at least from the current study that the blocking of pro-inflammatory cytokine (IL-1β and TNF-α) and induction of anti-inflammatory cytokine (IFN-γ, IL-12, and IL-4) might help to ameliorate the disease. We also have observed a higher abundance of IL-6 in many of our animal models which might modulate both pro- and anti-inflammatory pathways. Thus, understanding of a delicate balance between Th1 and Th2 cells and their secreting cytokines will be enlightening for DED research.

Our purpose for the study is to find a common mechanism through which one can treat DED, one of the predominant diseases of the ocular surface. We hope that this analysis will open new possibilities of treatment in addition to the existing broad treatment options. Besides, this analysis will also help us to understand the interplay of different cytokines in DED and we might recommend a combinatorial treatment for DED. This can act as a cautionary measure for designing a drug based on a single cytokine and might prevent previous issues such as the exacerbation of diseases in multiple sclerosis patients with anti-TNF treatment (10). DED is also a complex disorder and scientists are still optimizing the diagnostic markers of DED and it is important to have a combinational approach when the disease etiology of DED is not clear.

MATERIALS AND METHODS

Study Retrieval and Selection

The process for study selection is summarized in **Figure 1**. Databases like Medline, PubMed, Embase, Google Scholar, and Web of Science were searched systematically. Our final analysis is based on 53 studies filtered using well-defined parameters at each step. DED was searched with keywords for animal models – mice, rabbit, canine (dogs), porcine (pigs), rats, sheep, feline (cats), horse, guinea pigs, monkeys (non-human primates), knock-in, knock-out (KO) animals; immune cells, cytokines and chemokines – IL-6, TNF-α, IL-1β, IL-21, IL-17, CCR1, CCR2, CXCR3, CXCR2, IFN-γ, IL-23, $CD4^+$ T cells, $CD8^+$ T cells, Th17, Th1, Th2 cells, B cells, monocytes, macrophages, B-regs, IL-10, T-regs, HLA, TLR, IL-2, IL-4, Tc, IL-17, CCR6, CD45RO, CD45RA, CCR7, CCL20, CD11c, CD14, CD16, CD19, CD3, CD20, BAFF, APRIL, TACI, MyD88, IL-1, TNF, IFN, CCR5, CCL, MCP, MIP, CCR2, CX3CR1, IL-12, natural killer cells, IL-13, FoxP3, CD25, TGFβ, TGF, IL-14, IL-5, dendritic cell, complement system, neutrophils, memory cells, γδ-T cells, innate lymphoid cells, plasma cells, intra-epithelial $CD8^+$ lymphocytes, mucosal-associated invariant T cells, immunoglobulins (Igs), CD69, CD62L, CD103, M-CSF, GM-CSF, T-bet, GATA-3, CD44, CD45, CD27, CXCL10, CD40, CD28, IL-18, IL-1, TNF, IFN, and general terms interleukins, cytokines, antibodies, and lymphocytes as well. The exclusion process was first done by selecting articles according to title or abstract. Articles with patient participants and *in vitro* studies were excluded. Articles were then accessed fully, those without enough data, no immune factors included, or data that is not quantified, as well as no comparison to normal control, were excluded.

Animal Models

C57BL/6 mice used in the studies are induced to dry eye by exposure in a controlled-environment chamber upon subcutaneous injection with scopolamine hydrobromide (8, 9, 11–24), 0.2% benzalkonium chloride (BAC) induction (25), and environmental desiccating stress (26–30). Mice models with C57BL/6 background are Thrombospondin 1 (TSP-1) conditional knockdown mice (31, 32), CD25 conditional knockdown (33–36), PD knock-in mice (37) and B6.NOD-Aec1Aec2 mice (38, 39). Another wild type mice model (DS mice) is used to generate a dry eye model by applying desiccating stress (40, 41).

The *Aire* deficient mice model has a phenotype similar to SS symptoms by knocking out the *Aire* transcription factor that is responsible for self-antigen expression regulation (42, 43).

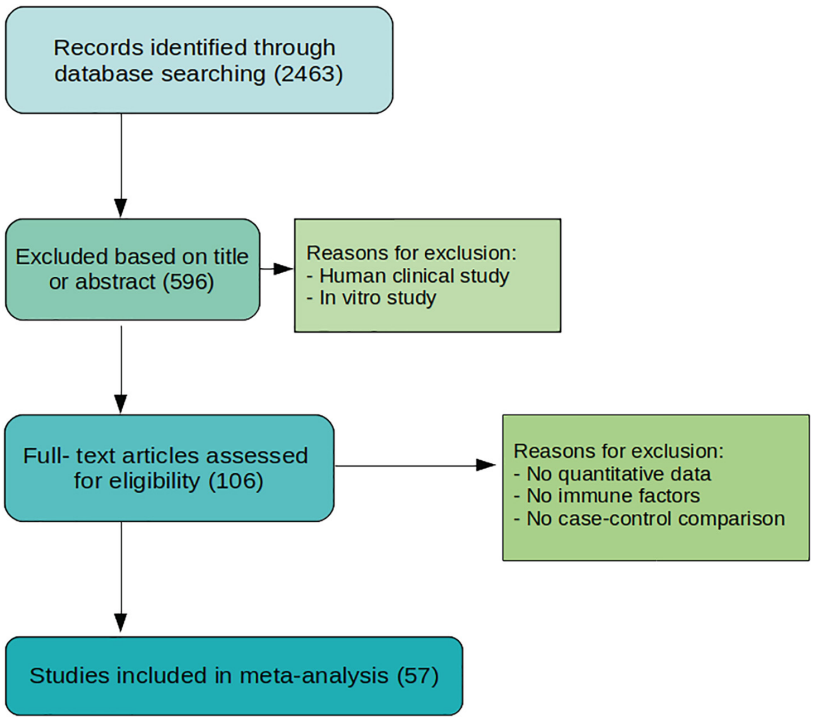

FIGURE 1 | Flowchart of study selection process. The process originates with 2463 studies from searching through several databases. A total of 57 studies are selected for meta-analysis after evaluation and exclusion.

Non-obese diabetic mice are immunodeficient mice that are prone to develop spontaneous autoimmune sialadenitis and exhibits SS (44–48). As an etiology, female mice develop autoimmune sialadenitis, whereas male mice develop dacryoadenitis and ocular surface inflammation. PSS is induced in NFS/N mice by performing thymectomy (49). CBA/J mice is a general-purpose model in which Botox-B (BTX-B) is injected to induce dry eye (50). In Albino Rabbit, 0.1% BAC eye drop is applied to induce dry eye (51–53). Another study used 1% atropine sulfate to instill into the eyes three times a day for 3 days (54). For Wister rats, Joossen et al., and Park et al., induced dry eye by removing the lacrimal gland (55, 56). Ru et al., induced dry eye by injecting scopolamine hydrobromide (57).

In Studies that used Lewis rats, Viau et al., and Han et al., induced the disease by injecting scopolamine hydrobromide (58, 59). Hou et al., induced the disease by injecting lacrimal gland extract from Sprague-Dawley rats to the Lewis rats (60). For Sprague-Dawley rats used in Hyun et al., the disease is induced by introducing Urban Particulate Matter (UPM) to the eyes (61).

In the analysis, these animal models are categorized into individual groups according to the animal strain. We have also performed the analysis categorizing the animal models according to the induction method to develop the DED (e.g., desiccating stress, Botox-B, benzalkonium chloride, UPM, and Atropine sulfate) but the overall result did not differ much (data not shown).

TABLE 1 | Excluded immune factors and the corresponding studies.

Study	Standard mean difference
De Paiva 2006_TNFα	812.90323
De Paiva 2010_TGFβ	9654.30464
De Paiva 2010_CCL20	68.96188
De Paiva 2010_IFNγ	60.12935
Yoon 2007_CCR5	478.87324
Yoon 2007_CCR3	−107.81889
Huang 2018_IL23R	54.19355
Huang 2018_IL21	894.19355
Huang 2018_CCL20	3603.87097
Huang 2018_IFNγ	180.64516
Joossen 2016_IL2	234.83871
Joossen 2016_IL12	1345.80645

Cases defined as outliers which the standard mean differences are higher than 50 or lower than −50.

Statistical Analysis

Data were analyzed using R package meta for meta-analysis. The script can be found on GitHub[1]. Github is an online repository used by bioinformaticians to store the data/code and this can be used by researchers in the future. Meta-analysis was performed for each of the animal models and the mean, standard deviation, and total number from the experimental and control group are analyzed from those animal models. To analyze all these

[1]https://github.com/yollct/animal_studies_dry_eye.git

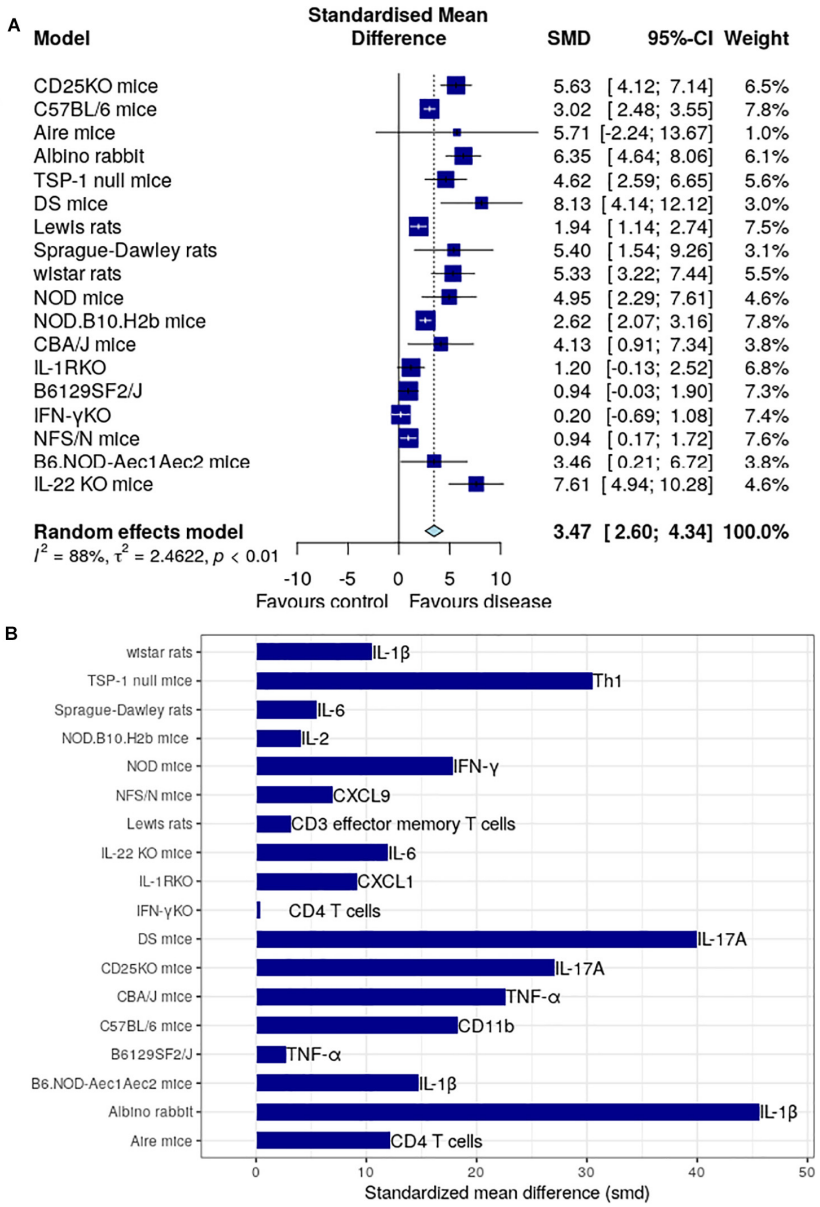

FIGURE 2 | Meta-analysis of different immune factors in animal models for dry eye disease. **(A)** The global standardized mean difference for each of the immune factors is obtained by our proposed meta-analysis is represented by the blue square in the forest plot. The 95% confidence interval is shown by the interval line across the blue squares. DS mice has the highest SMD among all animal models, followed by CBA/J mice with TNF-α. Forest plots for each of the models are shown in the **Supplementary Figures S1–S15**. **(B)** The standardized mean difference of immune factor for each model that is most expressed. IL-17A is the highest in DS mice and TNF-α in CBA/J mice.

data using meta-analysis models, a variance estimate telling how dispersed the data and effect size is required. The inverse variance (IV) method is the variance estimate that is taken inversely. IV weighting can resolve the inequality of the effect sizes among the studies by giving preferences to the larger effect size. To access the heterogeneity of the data, the I^2 value is calculated denoting the percentage of variability of the pooled effect sizes within the analysis. The data with I^2 values lower than 50% would be considered as coming from a homogeneous population, and the fixed-effects model would be used in this case; otherwise, the random-effects model was used. The fixed-effect model assumes the included studies have a higher variation, hence showing lower heterogeneity. These models resulted in a standard mean difference (SMD) with its 95% confidence interval (CI) for each group. Higher SMD indicates upregulation in the immune factors in animal models. Outliers with extreme effect sizes (>50 or <-50) are excluded to avoid distortion of effect estimates. The following **Table 1** shows the excluded studies.

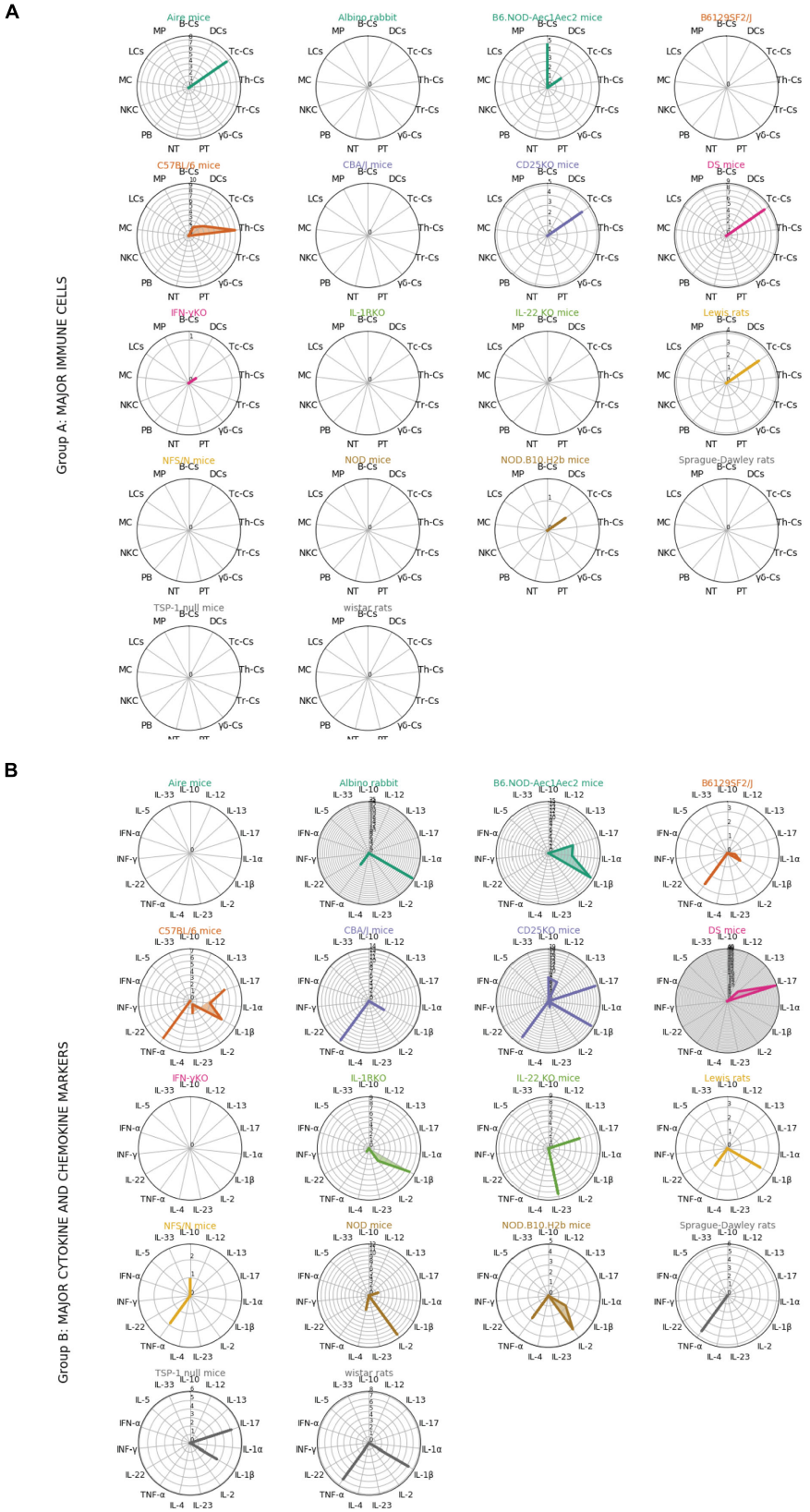

FIGURE 3 | Continued

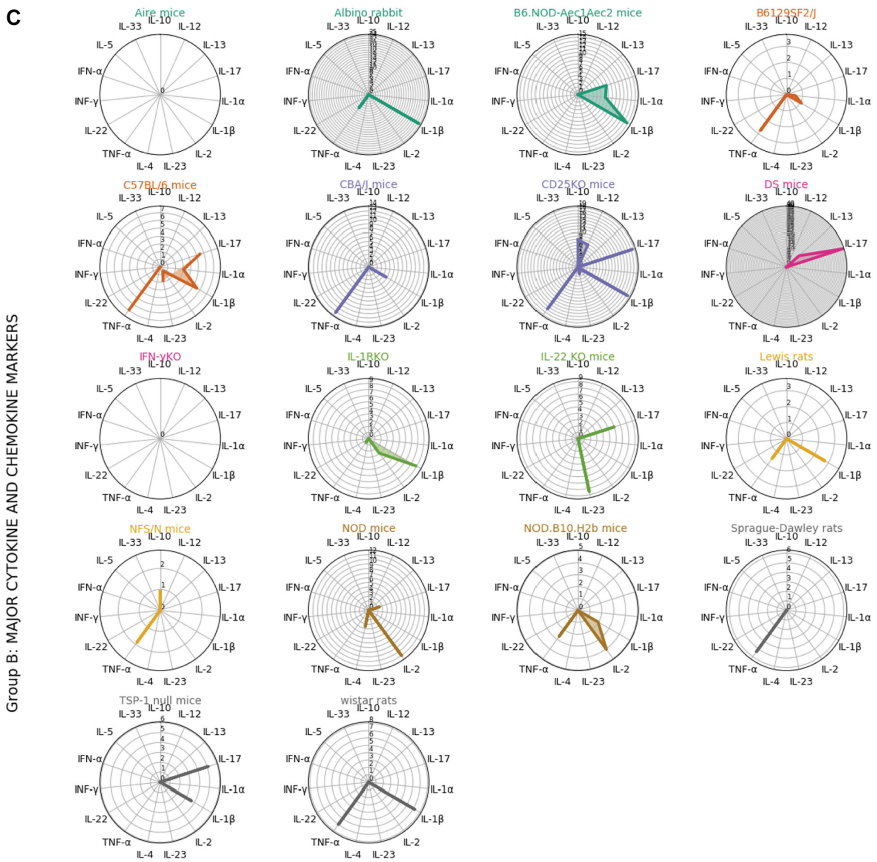

Figure 3 | Radar plots showing the relationships between each animal models and groups of immune factors. Immune factors are grouped into 3 groups (Group A, major immune cells; Group B, major cytokine and chemokine markers; Group C, functions overlying on multiple cell types). Each color represents different animal model. The standardized mean difference between disease model and control from the meta-analysis for each immune factors in different animal models are plotted in radar plots (see **Supplementary Material**). Each circle in the radar plots represents an increasing of 1 in SMD. Th-Cs, T helper cells; Tc-Cs, T cytoxic cells; Tr-Cs, T resident cells; gb-Cs, gb cells; NT, neutrophils; PB, plasmablasts; NKC, natural killer cells; MC, mast cells; LCs, langerhans cells; MP, macrophages; B-Cs:, B cells; DCs, dendritic cells; PT, platelets.

RESULTS

We have intensively studied 53 research papers, which were finalized for this specific study after a rigorous process of assessment shown in **Figure 1** and described in the section "Materials and Methods." **Figure 2** determines the animal model, which is mostly used, and the immune factor, which is predominant in these chosen animal models. **Figure 2A** shows the use of CBA/J mice (SMD: 7.28) mostly in the studies related with inflammatory molecule followed by Albino rabbit (SMD: 6.35), CD25-KO mice (SMD: 5.63), Sprague-Dawley rats (SMD: 5.40), Wistar rats (SMD: 5.33), NOD mice (SMD: 4.95), TSP-1 null mice (SMD: 4.62), C57BL/d mice (SMD: 3.08), B6.NOD-Aec1Aec2 mice (SMD: 2.92), Lewis rats (SMD: 1.94), and least shown with B6129SF2/J and NFS/N mice (SMD: 0.94). Besides, the AIRE mice and DS mice show quite higher SMDs (5.71 and 8.13, respectively) but that is due to the overpowering effects of two papers (40, 43) as shown in **Supplementary Figures 12**, **15**. Thus, we cannot consider these two animal models as the homogeneous distribution of the factors. The forest plots are described in detail in the **Supplementary Figures 1–15**. On the other hand, our observation shows a combination of pro-inflammatory and anti-inflammatory factors in those animal models. Among those molecules, IL-1β from Albino rabbit and Th1 type of immune cells from TSP-1 null mice are shown to be predominant (SMD: ∼45 and ∼30, respectively). This is followed by the presence of CD11b (SMD: ∼20) in environmental-factor-induced C57BL/6 mice models. In contrast, the environment-induced animal models (C57BL/6 an Albino rabbit) show the presence of innate immune cell features like dendritic cells (CD11b) and IL-6 (SMD: ∼20 for DED in Albino rabbit with BAC drops). Along with this, Th1-cell cytokine and chemokine IFN-γ, TNF-α, and CXCL9 seem to be quite predominant in the NOD (SMD: ∼20), CBA/J (SMD: ∼10), NFS/N (SMD: ∼5) mice, respectively. Besides, we could also find the presence of IL-1-cytokines in different other animal models (Wistar rats and B6.NOD-Aec1 Aec2 mice). As mentioned in the section "Materials and Methods," we have though seen higher SMD in the case of IL-17A for the DS and CD25-KO mice (SMD: ∼40 and ∼30, respectively), but the distribution of this immune factor is

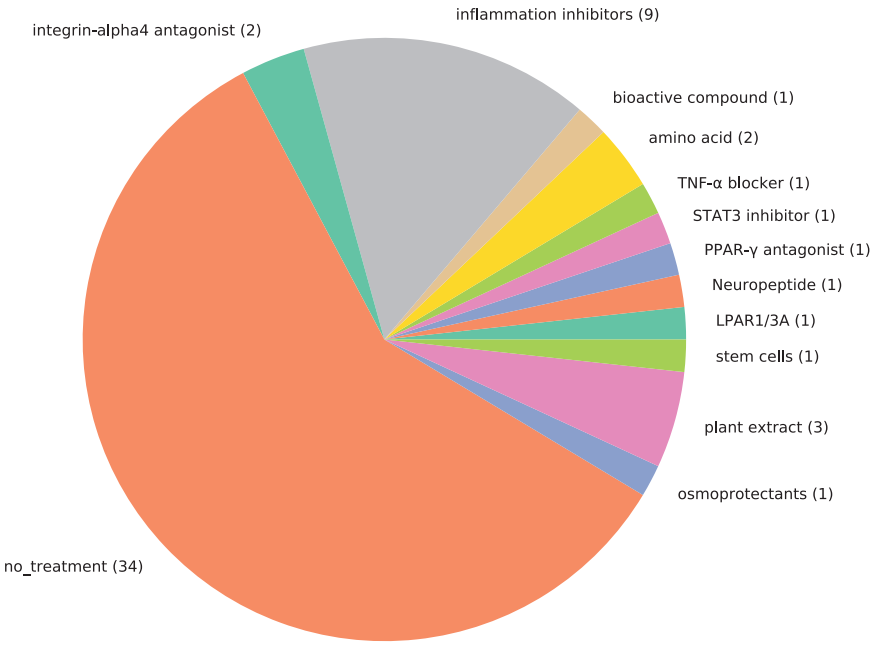

Figure 4 | Pie chart showing the proportion of studies that include treatment for dry eye disease. Each treatment used in the studies are represented by one color. The number in the pie chart shows the number of studies that used the type of molecules to evaluate treating effect. MK2I, MK2 inhibitors; LPAR1/3A, LPAR1/3 antagonists.

TABLE 2 | Studies contain treatment effect.

Study	Treatment	Type	Model
(26)	Betaine, L-carnitine, erythritol	Osmoprotectants	C57BL/6 mice
(9)	Pioglitazone (PIO)	PPAR-γ antagonist	C57BL/6 mice
(11)	Mixed medicinal plant extracts	Plant extract	C57BL/6 mice
(51)	Cyclosporine A (CsA)	Amino acid	Albino rabbit
(31)	Novel antagonist GW559090	Integrin-α4 antagonist	TSP-1 null mice
(12)	Corticosteroids, doxycycline	Inflammation inhibitors	C57BL/6 mice
(52)	Epigallocatechin gallate (EGCG), hyaluronic acid	Inflammation inhibitors	Albino rabbit
(61)	Amygdalin	Bioactive compound	Sprague-Dawley rats
(61)	Apricot kernel extract	Plant extract	Sprague-Dawley rats
(15)	HL036	TNFα blocker	C57BL/6 mice
(55)	Cyclosporine A (CsA)	Amino acid	Wistar rats
(55)	Restasis and dexamethasone	Inflammation inhibitor	Wistar rats
(17)	Novel antagonist GW559090	Integrin-α4 antagonist	C57BL/6 mice
(44)	Topical TSG-6	Inflammation inhibitors	NOD.B10.H2b
(73)	Adiponectin	Inflammation inhibitors	C57BL/6 mice
(45)	Vasoactive intestinal peptide (VIP)	Neuropeptide	NOD mice
(50)	FK506	Inflammation inhibitors	CBA/J mice
(46)	Ki16425	LPAR1/3A	NOD mice
(56)	Polygonum cuspidatum (PCE)	Plant extract	Wistar rats
(25)	S31-201	STAT3 inhibitor	C57BL/6 mice
(57)	α-Melanocyte-stimulating hormone	Inflammation inhibitors	Wistar rats
(54)	CM-hUCESC	Stem cells	Albino rabbit
(53)	Epigallocatechin gallate (EGCG), hyaluronic acid	Inflammation inhibitors	Albino rabbit
(22)	MK2i	Inflammation inhibitors	C57BL/6 mice

This table shows the molecules and the model used by studies that tested the effect of molecules as a mean of treatment.

not homogeneous. It is mostly the overpowering effects of the few papers (36, 40) as clearly shown in **Supplementary Figures 3**, **15**.

In the radar plots of **Figure 3**, we followed a cluster analysis with three different groups of factors: group A with major immune cells, group B with major cytokine and chemokine markers, and group C with the functions overlaying on multiple cell types. We have only mentioned the significantly modulated factors in the section "Results" to avoid further complications. Some examples for group C are TLR4, IL-6, CD45, which are involved in both innate and adaptive immune system pathways. The radar plot in **Figure 3** Group A depicts that $CD4^+$ and $CD8^+$ Th-cells play a predominant role in C57BL/6, B6.NOD-Aec1Aec2, Aire mice, DS mice, CD25-KO, and TSP-1 null mice. Apart from two Th cells, B cell is also shown to be important in this cluster represented by the TSP-1 null and B6.NOD-Aec1Aec2 mice. Some mice model radars in this cluster are empty reflecting the importance of specific analyzed populations in **Figure 3** Group B. Here, we mostly observe the importance of Th1 cytokines IL-1β and TNFα represented by the Albino rabbit, Lewis rats, Sprague-Dawley rats, CD25-KO, B6 NOD-Aec1Aec2, B61295F2/J, C57BL/6, CBA/J, NFS/N, NOD, and TSP-1 null mice. The animal models influenced by the environmental factors can have the presence of both the cytokines related to the innate (IL-1β) and adaptive immune system (IFN-γ, TNF-α, IL-17, and IL-23) as depicted in the Albino rabbit and C57BL/6. Besides, the other T-cell types (Th17 and Tregs) shown to play a role in the B6.NOD-Aec1Aec2, C57BL/6, CD-25-KO, DS, NOD, NFS/N, TSP-1 null mice were represented by IL-17 and IL-10. In our calculation, we could mostly find the abundance of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-2) with few animal models showing the anti-inflammatory cytokines (IL-10 and IL-4). Our finding of the predominance of Th1-related cytokines did not alter much in **Figure 3** Group C. We could mostly find the importance of cytokine IL-6 in this cluster followed by TGF-β and CXCL9. We observe the presence of CD11b and TLR4 in C57BL/6 mice, which are an important component of the innate immune system.

To find the treatment molecule used in the selected papers, we showed **Figure 4** and **Table 2**. As treatments related to the immune factors, we could see the use of anti-inflammatory drugs corticosteroids, doxycycline, TNF-α blocker HL036, and immunosuppressant MK2i (8, 10, 17). Other than the immune factors, the treatment paradigm includes osmoprotectants (betaine, L-carnitine, and erythritol), amino acid (cyclosporine A), integrin-α4 antagonist (dexamethasone), artificial tears (epigallocatechin gallate (EGCG), hyaluronic acid), plant antioxidant (10, 17, 26, 32, 51, 53). As it is shown from **Figure 4** that the number of studies has not predominated in any case. It is a hint that a combinational treatment might be helpful in alleviating DED. Our effort here is not to find the list of treatments available in the literature but to show that there are still unspecific treatments (e.g., immunosuppressants, plant extract), which are recommended for this complex ocular surface disease. Thus, there is a huge gap in knowledge and treatment regimens for DED.

CONCLUSION AND DISCUSSION

The functionality of $CD4^+$ Th1 and Th2 subtypes are related to the presence of various cytokines. Detection of IL-2, IFN-γ, IL-4, and IL-5, associating with B cell accumulation, suggests a role of Th1 in disease induction and maintenance, and Th2 in disease progression. The Th1-associated pro-inflammatory cytokine IFN-γ is regulating the conjunctival apoptosis in desiccating stress models (62–65), and IL-7 upregulates the expression of IFN-γ (63). IL-13 as a Th2 cytokine is also proposed to be involved in disease pathology as shown in $Id3^{-/-}$ mice (66). The role of Th17 cells was critically explored in SS in a chronic dry eye mouse model. Chronic ocular surface damage is mainly mediated by a memory T cell population, the response of which is predominantly mediated by Th17 cells (12). Co-transfer of $CD8^+CD103^+$ Treg had no effect indicating that $CD8^+$ can suppress the initiation of pathogenic Th17 cells, but not the prolongation of disease (41). In our observation, we have found the involvement of the adaptive immune system pathways in all the animal models but the presence of innate immune system pathways only in the environment-induced animal models. This is a piece of important information while designing a drug for the treatment of DED. Current drugs for DED, which are in the Human study phase 2/3 clinical trials, are mostly related to the innate immune system pathways (67). This study enables us to identify the importance of the right environmental condition in which the molecules/cell types of adaptive immune system pathways are involved. Along with the essential balance between Th1 and Th2 cells with the pro- and anti-inflammatory cytokines, it is also interesting to note that IL-6 and TGF-β are promoting cytokines for Th17 cells, and therefore although by themselves are considered to be general, by blocking either or both of those, there will be considerable inhibiting effects downstream on the Th17 cells (68).

This study is also a trial to increase the awareness of the researchers working in this field. Awareness is twofold here: at first, there is no single or combination of studies in DED that depicts the exact picture of the modulation of several cytokines. This representation of the cytokine modulation is shown with a promising approach in detail in the case of corneal transplantation by Reza Dana et al., using several animal models. They have shown the beneficial effects of low-dose IL-2 and IL-6 blocking antibody in their previous papers (69, 70). Secondly, after understanding of the involvement of the cytokines in several conditions of the diseases, one can predict/design a drug, using one cytokine or combination of the cytokines. This will certainly be a much safer option and will not just follow the mainstay of the treatment which in many cases is not safe and effective. One can take an example from anti-TNF treatment where 40% of patients have no response to the treatment and it is associated with some adverse effects like increased risk of infection, triggering of development of autoimmune diseases due to the global inhibition of TNF biological functions (71).

This study is a conglomeration of the observations from the publications of the last 20 years and gives a hint to the research direction in the field of ocular surface disorders. The number of references we have worked on did not represent a huge number (52) – this can be one of the limitations of the study. But, the number of animal models we have is relatively high (14) along with the diversity in different species following our search criteria. Another important point to note is that we only considered those studies for the therapeutic approach where the drugs are tested i*n vivo* for identification of immunological parameters. In this case, we have not included those *in vivo* studies where the animal models are only used for the tolerability assay analysis like the testing of Xiidra in pigs (72). Despite these two limitations, this study gives an indication which immune cells or which immune mediators are able to alleviate the ocular surface diseases. There are already existing conditional knockdown animal models (35, 41) with the deletion of genes for the important cytokine and chemokine factors like IL-1β and IFN-γ discussed in the manuscript and combinational treatment in these animal models along with dry eye conditions will give new insight to the field of research. The preponderance of the adaptive immune system factors in animal models is different than what we have observed from the meta-analysis of the human patients where we have found the predominance of dendritic cells (innate immune system) (7). It is true though that the dendritic cells are considered to be the part of the innate immune system, this is also an important cellular component to drive the adaptive T cells responses through their presentation of the antigens. What we are mentioning here is that our meta-analysis result is different between the human studies and animal models. This is a word of caution that no animal models are an exact representation of human diseases and depicts the challenge of the researchers involved in representing the multi-factorial human diseases.

SUMMARY

- DED animal models mostly show the predominance of Th1-modulatory and pro-inflammatory cytokines (IL-1β and TNF-α) despite the modulation either genetically or environmentally.
- It is clear at least from the current study that the blocking of pro-inflammatory cytokine (IL-1β and TNF-α) and induction of anti-inflammatory cytokine (IFN-γ, IL-12, and IL-4) might help to ameliorate the disease. An understanding of the delicate balance between Th1 and Th2 cells and their relationship with the pro- and anti-inflammatory cytokines will enrich the DED research.
- In the animal model induced by environmental factors, the innate immune pathway may play a more dominant effect than the adaptive immune system pathway.
- IL-17 though has shown to be important, but the distribution of this factor is not homogeneous among all the research studies. It can be an over-powering effect from a few selected papers. But an interesting point to note is that an understanding of the co-operative mechanism between the cytokines especially IL-6, TGF-b, and Th17 will foster DED therapy research.
- This study gives an insight into the treatment paradigm for chronic and acute DEDs in terms of the identification of immune factors in the autoimmune and environmental factor-induced animal models. This study also tries to identify the gap of knowledge in the specific therapeutic options for the DED.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

TB designed the study and wrote the manuscript. CL did all the analysis and figure preparation. SD critically commented and helped in revising 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.2020.01930/full#supplementary-material

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