CHARACTERIZATION, FUNCTIONS, AND ROLES OF ANTIGEN-SPECIFIC REGULATORY T CELLS IN HEALTH AND DISEASE

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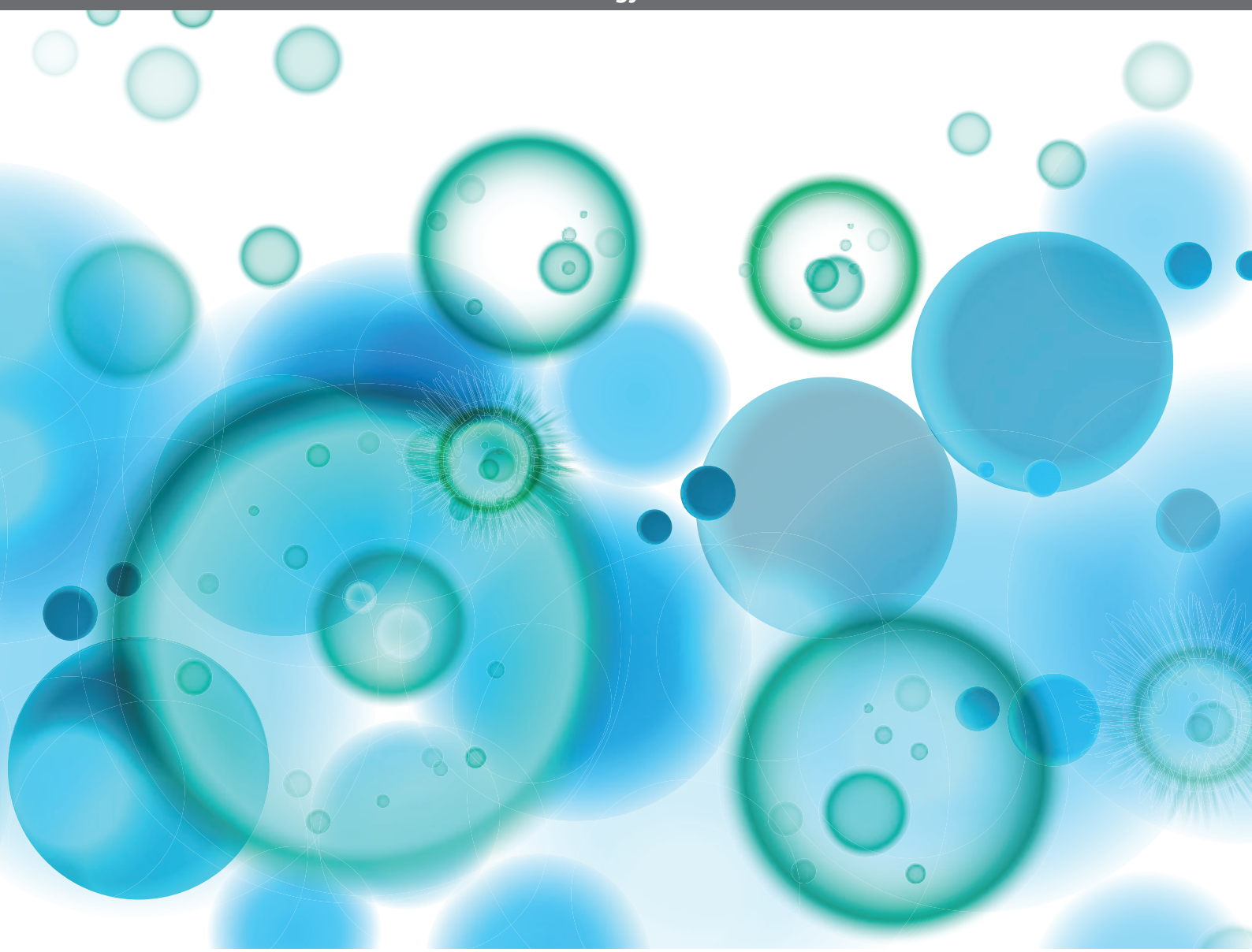

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CHARACTERIZATION, FUNCTIONS, AND ROLES OF ANTIGEN-SPECIFIC REGULATORY T CELLS IN HEALTH AND DISEASE

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Editorial: Characterisation, functions and roles of antigen-specific regulatory T cells in health and disease

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Treg - regulatory T cell, antigen-specificity, immunotherapy, immune tolerance, Foxp 3

Editorial on the Research Topic
Characterization, functions, and roles of antigen-specific regulatory T cells in health and disease

Introduction

The control of immune responses against self and how to induce immune tolerance has intrigued immunologists for nearly 75 years. The clonal deletion theory of Burnet (1) received unquestioning support and is still a partial explanation for lack of reactivity to self.

A decade after the discovery that lymphocytes produced by the thymus are key to immune responses (2), Gershon et al. described thymus derived suppressor cells (3) and these cells were implicated in prevention of autoimmunity and transplant tolerance (4). In addition, neonatal and adult thymectomy promote the development of autoimmunity (4) which could be suppressed by transfer of normal lymphocytes (5). Studies on suppressor cells identified they were a subset of $CD8^+$T cells that expressed I-J, a molecule associated with "Ia" in MHC (5). Cloning of mouse MHC found no gene for I-J in MHC of mice (6) and suppressor cells were declared non existent (7). The word "suppressor cells" was eliminated from the immunological dictionary.

From near extinction, suppressor cells that expressed CD4 not CD8 were discovered in the mid 1980 by Hall et al. and their activity was antigen-specific (8). These $CD4^+$ regulatory cells had markers of activated/memory T cells, including the IL-2 receptor CD25, expression of lower molecular weight forms of CD45, and MHC class II (9). Furthermore, these cells are highly dependent on specific-antigen and cytokines to survive and expand (10) but IL-2 alone did not promote their survival. It was for this reason, Hall et al. looked for CD25 expression on antigen-specific suppressor cells. These

antigen-specific T suppressor cells had other characteristics of an activated memory cells (11) in that they did not recirculated from blood to lymph (12). The inability of IL-2 alone to sustain activated antigen-specific $CD4^+CD25^+$T cells, led us to investigate the role of other cytokines in activation and maintenance of $CD4^+CD25^+$Treg (13, 14). We found that naïve Treg proliferated when cultured with either IL-2 or IL-4 in the presence of antigen acquired increased potency to suppress *in vitro* and *in vivo* (13).

Whilst these findings from the 1980s lay unrecognized, two studies reinvigorated research on regulatory cells. First the demonstration of infectious tolerance mediated by $CD4^+$ T cells (15). Second, Sakaguchi's application of Hall's findings on activated $CD4^+CD25^+$ T cells to show they also prevent autoimmunity (16). The title of their paper starts as "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25)." indicating they accepted our premise that $CD4^+CD25^+$ T cells were activated $CD4^+$T cells. It is now clear the cells they described were resting, thymus derived cells whose TCR recognize autoantigens. These cells express FoxP3, the transcription factor that maintains regulatory function of Tregs (17). How naïve resting thymic derived Treg with no antigen specificity are activated to antigen-specific Tregs remains an unfolding puzzle. The potential of antigen specific Tregs as therapy in autoimmune disease and transplantation was rapidly appreciated and remains a subject of active investigation.

Articles in this Research Topic illustrate the wide range of studies being undertaken to improve our understanding of how antigen-specific Treg are activated, can be monitored, expanded and their potential application for therapies.

The role of cytokines in Treg activation is discussed in several papers. Bhaskaran et al. investigated the role of IL-1β -MyD88-mTor in activation of Th17-like Treg. MyD88 deletion in $FoxP3^+$ T cells alters its function and results in increased mucosal infection and inflammation which coincided with the reduction of IL-17A expressing $FoxP3^+$ (Treg17) and increased Treg IFN-γ. Hall et al describe how IL-5 therapy promotes the generation of a unique population of antigen-specific Tregs that is highly potent in prevention of allograft rejection. These cells are called Ts2 and have upregulation of IL-5Rα.

Iwaszkiewicz-Grzes et al described epigenetic changes with antigenic stimulation of Treg. They show Treg activation by specific antigen alters their functional capacity.

Shimojima et al. studied Treg stability in antibody associated vasculitis, showing inhibition of activated Tregs by oxidative stress, reinforcing the negative effect inflammation has on Treg function. The mediator was reactive oxygen species (ROS) acting *via* phosphorylated mammalian target of rampamycin (mTOR).

Shevyrev et al. reviewed the interaction of recognition and presentation in activation of T cells, including conversion of effector cells to regulatory cells.

The complexities of growing antigen-specific Treg are highlighted in three articles. Lee et al. described how they expand high potency human alloreactive Treg *ex vivo*. They compared expansion efficiency and characteristics of *ex vivo* expanded human Treg generated by stimulation with either, allogeneic stimulated B cells (sBcs) or matured monocyte derived dendritic cells (sDCs). Both protocols induced a similar Treg phenotype, but sDCs expanded twice the number of Treg. These findings establishe that sDCs stimulation is a viable option for alloreactive Treg expansion.

Cortes-Hernandez et al. described how antigen-specific Treg can be expanded from patients with renal failure. This study showed purification of alloantigen specific Treg from chronic kidney disease patients and their successful long-term expansion that maintained their suppressive phenotype and function.

Therapeutic potential of antigen-specific Treg is demonstrated in several papers. To circumvent the complexities of growing natural Treg, Muller et al. detailed methods to genetically engineer anti-HLA-A2 regulatory T cells as potential inducers of transplant tolerance. HLA-A2 mono-specific CAR Treg maintained Treg phenotype and function *in vitro* and *in vivo* as they selectively homed to HLA-A2 expressing islets grafts.

Selck and Dominguez-Villar reviewed approaches to generate antigen-specific Tregs by genetic engineering of antigen-specific T effector cells or polyclonal Tregs, and activation of Tregs *in vivo*.

The roles of activated Tregs in diseases such as pulmonary hypertension(Tian et al.) and therapy for food allergy (Liu et al.), milk intolerance (Zhang et al.), are reviewed with discussion of many important aspects of using antigen-specific therapies in autoimmune disease including using combined strategies and tissue specific targeting (Serr et al.). Hu et al. reviewed the role of antigen-specific Tregs in renal transplantation (Selck and Dominguez-Villar) .

Hu et al described cells that do not share standard markers of Treg but exhibit regulatory functions. They studied a possible mechanism of induction of previously described $TCR\alpha\beta^+$double negative T cells (DNT) from $CD4^+$ T cells by stimulation with immature DCs.

Liu et al studying chronic hepatitis B patients showed expression of NKG2D on iDNT cells enhanced their regulatory function of limiting proliferation and survival of B cells. IL-35 dependent T cells regulation by regulatory $IL\text{-}35^+$B cells within classical $CD19^+CD24^{hi}CD38^+$Breg is described in chronic hepatitis B, and was dependent on cell-to-cell contact controlling IFN-γ producing $CD4^+$ and $CD8^+$T cells.

Lyu et al. used single cell RNA sequencing and TCR sequencing to assess the functions of $CD4^+$ T cell subsets, including Treg and their interactions, during CMV infection. Treg phenotype during CMV infection showed markers that are proinflammatory, inhibitory, chemokine receptors and cytotoxic related markers in addition to characteristic markers of Treg.

This suggests clustering of these cells in a self-sustaining positive feedback loop.

These studies are a small part of international endeavours directed at turning an orphan and once dismissed cell into 'Cinderella' which may have wide application and unique effects that could cure a number of autoimmune diseases and aid in the prolongation of transplanted tissues.

Author contributions

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

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Conflict of interest

The 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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IL-1β-MyD88-mTOR Axis Promotes Immune-Protective IL-17A$^+$Foxp3$^+$ Cells During Mucosal Infection and Is Dysregulated With Aging

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CD4$^+$Foxp3$^+$T$_{regs}$ maintain immune homeostasis, but distinct mechanisms underlying their functional heterogeneity during infections are driven by specific cytokine milieu. Here we show that MyD88 deletion in Foxp3$^+$ cells altered their function and resulted in increased fungal burden and immunopathology during oral *Candida albicans* (CA) challenge. Excessive inflammation due to the absence of MyD88 in T$_{regs}$ coincided with a reduction of the unique population of IL-17A expressing Foxp3$^+$ cells (T$_{reg}$17) and an increase in dysfunctional IFN-γ$^+$/Foxp3$^+$ cells (T$_{reg}$IFN-γ) in infected mice. Failure of MyD88$^{-/-}$ T$_{regs}$ to regulate effector CD4$^+$ T cell functions correlated with heightened levels of IFN-γ in CD4$^+$ T cells, as well as increased infiltration of inflammatory monocytes and neutrophils in oral mucosa *in vivo*. Mechanistically, IL-1β/MyD88 signaling was required for the activation of IRAK-4, Akt, and mTOR, which led to the induction and proliferation of T$_{reg}$17 cells. In the absence of IL-1 receptor signaling, T$_{reg}$17 cells were reduced, but IL-6-driven expansion of T$_{reg}$IFN-γ cells was increased. This mechanism was physiologically relevant during *Candida* infection in aged mice, as they exhibited IL-1 receptor/MyD88 defect in Foxp3$^+$ cells, loss of p-mTOR^{high}T$_{reg}$17 cells and reduced levels of IL-1β in oral mucosa, which coincided with persistent tongue inflammation. Concurrent with T$_{reg}$ dysfunction, aging was associated with increased CD4$^+$ T cell hyperactivation and heightened levels of IL-6 in mice and humans in oral mucosa *in vivo*. Taken together, our data identify IL-1β/MyD88/T$_{reg}$ axis as a new component that modulates inflammatory responses in oral mucosa. Also, dysregulation of this axis in an aging immune system may skew host defense towards an immunopathological response in mucosal compartments.

Keywords: T$_{reg}$, Foxp3, T$_{reg}$17, IL-1β, *Candida*, fungal infection, senescence, aging

INTRODUCTION

$CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{regs}) are central in controlling the magnitude of an immune response thereby regulating autoimmunity and maintaining mucosal tolerance (1). We and others have shown that 5%–10% of $CD4^+$ T cells have a T_{reg} phenotype in normal oral mucosa (2–4). Molecular components that define their functional plasticity and heterogeneity are not completely characterized during mucosal infections, and appear to be driven by specific stimulation and cytokine milieu. *Candida* is an innocuous commensal in >60% of human population but causes opportunistic infections and chronic oral erythematous candidiasis in elderly individuals (5). Host pathogen recognition receptors including toll-like receptor (TLR)-2, Dectin, and EphA2 are known to recognize *Candida* (6, 7). C-type lectin receptor-Syk (spleen tyrosine kinase) adaptor CARD-9-IL-1β axis, IL-17 receptor signaling, and Th17 cells play important roles in antifungal immunity (8, 9). T_{regs} are critical for enhancing early Th17 host responses, as well as controlling excessive immunopathological responses during the resolving phase of oropharyngeal candidiasis (OPC). While thymic T_{regs} (tT_{regs}) regulate systemic Th1 autoimmunity, peripheral T_{regs} (pT_{regs}) are generated extrathymically at mucosal interfaces and control commensal microbiota composition and local inflammation (10, 11). Microbial stimulants are known to control pT_{reg} functions and the mechanisms have begun to be elucidated (12–14). Some studies imply that T_{reg} suppression can be bypassed by microbial signals such as toll-like receptor (TLR) ligands, myeloid differentiation primary response 88 (MyD88) signals, and pro-inflammatory cytokines (15–17). Others conclude that MyD88 and cMAF dependent microbial sensing by T_{regs} are shown to enhance their suppressive capacities (2, 18–23). Thus, the intrinsic role of MyD88 in mucosal T_{regs} during an infection remains to be defined. Here we show that IL-1β/MyD88 principally promotes the induction and proliferation of $ROR\gamma t^+IL\text{-}17^+Foxp3^+$ cells ($T_{reg}17$) in an mTOR dependent manner during *Candida* challenge. These cells are required for optimal resolution of infection and inflammation. Absence of IL-1β signaling in $Foxp3^+$ cells also leads to an IL-6 driven expansion of T_{reg}IFN-γ cells, which appear to coincide with immunopathology. While RORγt expressing $Foxp3^+$ cells have been implicated in playing diverse roles in intestinal inflammation (13, 24, 25), our results demonstrate their immune-protective functions and the contrasting roles of IL-1β and IL-6 in determining their plasticity and function during an oral mucosa infection. Our data also highlight an age dependent dysregulation of this mechanism due to an imbalance in these cytokines. Collectively, these results demonstrate that IL-1β/MyD88 signaling augments T_{reg} functions and modulates mucosal immunity and also provide new insights in to a mechanism underlying immune-dysfunction in human aging and mucosal infections.

Abbreviations: CA, *Candida albicans*; CLN, Cervical lymph nodes; FYcre, $Foxp3^{YFPcre}$ mice; HKGT, Heat killed *Candida albicans* germ tube; HOIL, Human oral intra-epithelial and lamina propria leukocytes; MFYcre, Mice with $Foxp3^+$ cell specific deletion of MyD88; MyD88 crossed with $Foxp3^{YFPcre}$; MyD88, Myeloid differentiation primary response 88; MOIL, Mouse oral intra-epithelial and lamina propria leukocytes; OPC, Oropharyngeal candidiasis; SPLN, Spleen; T_{reg}IFN-γ, IFN-γ expressing dysfunctional $Foxp3^+$ cells; $T_{reg}17$, IL-17A expressing $Foxp3^+$ cells.

MATERIALS AND METHODS

Mouse Cells, Patients, Human PBMC, and Gingival Biopsies

Mouse experiments were performed at Case Western Reserve University (CWRU) under an approval from the CWRU Institutional Animal Care and Use Committee, and followed all guidelines and regulations. Some of the experiments were also done at NIAID, NIH in compliance with the NIAID Institutional Animal Care and Use Committee's guidelines and under an approved protocol. Young (6-9 weeks of age) $Myd88^{flox/flox}$, $Foxp3\text{-}YFP^{cre}$ transgenic mice, BALB/cJ, C57BL/6J, $Foxp3^{GFP}$ reporter, CD45.1 congenic mice, and $IL\text{-}1R^{-/-}$ mice, as well as aged (12–18 months of age) C57BL/6 mice were purchased from Jackson Laboratories. Animals of both genders were used for experiments. Foxp3 specific-MyD88 deficient mice (MFYcre) were generated by breeding $Myd88^{flox/flox}$ and $Foxp3\text{-}YFP^{cre}$ (FYcre) mice. Human PBMC, gingival biopsies and saliva were obtained under a protocol approved by the University Hospitals Cleveland Medical Center Institutional Review Board. Informed consents were obtained from participants after the nature and all possible consequences of the study were fully explained to them. Healthy subjects were 18 years of age and older and in good general health. Exclusion criteria were follows: oral inflammatory lesions (including gingivitis and periodontitis), oral cancer diagnosis, soft tissue lesions, and the use of tobacco in the past month. Single cell suspension of MOIL and HOIL were prepared after Collagenase 1A digestion of the mouse tongue/palatal/gingival tissues and human gingival biopsies, respectively.

Antibodies and Reagents

Purified or fluorochrome conjugated mouse and human α-CD3 (145-2C11), α-CD28, α-CD25 (3C7 and 7D4), CD4, IL-2, IFN-γ, IL-17A, TNF-α, Foxp3, CD45, CD8, CD11C, CD38, HLADR, Phospho-p70 S6 Kinase (Thr389), Phospho-Akt 1 (Ser473), IL-10 (JES5-16E3), IL-6, and p-mTOR antibodies, carboxyfluorescein diacetate succinimidyl ester (CFSE), and Cell Proliferation Dye eFluor 670 (CPD-670) were all purchased from Life Technologies/Thermofisher. PE conjugated F4/80 Monoclonal Antibody (BM8), PerCP-eFluor 710 conjugated Ly-6G Monoclonal Antibody (1A8-Ly6g), APC conjugated CD11b Monoclonal Antibody (M1/70) were all purchased from Ebiosciences/Thermofisher Scientific. Recombinant IL-1β was purchased from BioBasic Inc (Amherst, NY). Human TGF-β1 was purchased from R&D systems. Anti-mouse CD121A (IL1R1) BV421 (1F3F3D4) was purchased from BD Biosciences. Anti-mouse blocking IL-1β blocking antibody was bought from Novus Biologicals. Anti-MyD88-PE antibody was purchased from Santacruz biotechnologies. $CD4^+$T cell isolation kit II (Miltenyi Biotec, Auburn) was used for purification of $CD4^+$ cells, which were further flow cytometry sorted for naive cells. In some experiments, we used flow cytometry-sorted $CD4^+CD25^+GFP^+$

T_{reg} cells or $CD4^+CD25^-$ GFP^- responder cells from $Foxp3^{GFP}$ reporter mice. The purity of $CD44^{lo}CD62L^{hi}CD25^+$ naive cells was more than 98%. CD4, CD4 naïve cell and T_{reg} magnetic isolation kits were also used and were purchased from Stem cell Technologies (Vancouver, Canada). Mouse cells were cultured in complete RPMI-1640 (Hyclone) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Some *in vitro* experiments were done using the X-VIVO-15 serum-free media from Lonza/Biowhittaker. Mouse IFN-γ and TNF-α ELISA kits were purchased from Ebiosciences/Thermofisher Scientific. IL-1β and IL-6 ELISA kits were from Boster Bio (Pleasanton, CA). Heat killed *Candida albicans* germ tubes (HKGT) were generated in the laboratory by heat killing the germ tubes at 75°C for 60 min. Germ tubes were prepared by growing blastospores (10*9/ml) in complete RPMI-10 at 37°C with CO_2 for 4–6 h, or until the budding of germ-tubes.

Cell Stimulation *In Vitro*

Cells from SPLN, CLN, and MOIL were stimulated in U-bottom 96 well plates using 1 μg/ml of plate-bound α-CD3 and 2 μg/ml α-CD28 antibodies with IL-1β (1–10 ng/ml), TGF-β1 (2 ng/ml), and HKGT for 3–6 days, as indicated. $CD90^+$ T cell depleted splenocytes were added as antigen presenting cells (APC), at a T cell: APC ratio of 3:1 during the initiation of cultures, when indicated. In some experiments, $CD4^+$ T cells were pre-labeled with CPD-670 before adding in cultures to assess their proliferation. For co-culture T_{reg} suppression assay, CPD670 labeled $CD4^+CD44^{lo}CD62L^{hi}CD25^+$ naive responder T (T_{resp}) cells (3×10^4) were stimulated in U-bottom 96-well plates with 3×10^4 control $CD4^+CD25^-$ cells or 3×10^4 T_{reg} cells using soluble 1 μg/ml α-CD3 and 2 μg/ml α-CD28 antibodies (26).

Quantitative-Reverse Transcriptase PCR (q-RT PCR)

Naïve $CD4^+$ T cells were stimulated as above with soluble 1 μg/ml α-CD3 and 2 μg/ml α-CD28, TGF-β1, HKGT with or without IL-1β for 3 days and were used for q-PCR analyses of ROR-γt, Foxp3, IL-17A, and IFN-γ mRNA. RNA was isolated using an RNA isolation Kit (BioBasic). Removal of genomic DNA from purified RNA was done by DNase (Ambion). Mu-MLV reverse transcriptase, oligo-dT primers (BioBasic), and SYBR Green PCR Kit (BioBasic) and real time PCR machine (Applied Biosystems) were used for cDNA synthesis and qPCR. All primers for PCR (BioBasic) were designed to amplify a coding region within a single exon. The relative amount of mRNA of interest was estimated from its Ct values, which were normalized to the β-actin mRNA levels, assigning values of "1" to unstimulated or "day 0" $CD4^+$ T cells that were used as control samples.

Immunohistochemistry of Proteins and Intracellular Staining of Cytokines

For immunocytochemical periodic acid schiffs (PAS), hematoxylin and eosin (H&E), and Foxp3 antibody histological staining, tongue tissues were cleaned and rinsed with PBS, fixed with 10% formalin overnight, and rehydrated in 70% ethanol overnight. This was followed by sectioning and staining of paraffin sections by the commercial facility (Histoserv, Inc, MD). For single-cell flow cytometry staining, cells were cultured as above and washed in PBS or PBS/BSA before surface staining using the antibodies. For Foxp3 staining, the cells were fixed with Foxp3 fix-perm set (eBioSciences/Thermofisher) after surface staining. Live-Dead viability staining was used to remove dead cells in the analyses. Appropriate un-stain, isotype, secondary antibody, single stain and FMO controls were used. Before intracellular cytokine staining, cultures were re-stimulated with PMA (50 ng/ml) and Ionomycin (500 ng/ml) for 4 h, with brefeldin-A (10 μg/ml) added in last 2 h. For p-IRAK, p-Akt, p-mTOR, and p-70-S6K staining, the cells were washed, fixed and were stained with Phosflow staining kit from BD Biosciences using manufacturer's protocol.

Flow Cytometry and Confocal Microscopy

Data was acquired using BD Fortessa cytometers and were analyzed using FlowJo 9.8 or 10.5.3 softwares. Cells were cytospun on the slides, fixed, permeabilized for intracellular flow cytometry and confocal staining.

Oral *Candida* Infection

Mice were infected as previously described (27, 28). Briefly, they were sublingually infected with tongue abrasion and under anesthesia by placing a 3 mm diameter cotton ball saturated with 1×10^7 *Candida albicans* (SC5314) blastospores for 90 min. Mice were re-infected on day 14 or 15 after primary infection for assessing the secondary immune responses *in vivo*. Mouse body weight was monitored every day until sacrifice. Tongue inflammation scores were assessed as follows: 0 = No fungus and immune infiltrates; 1 = Sparse immune infiltrates; 2 = Sparse fungus with low immune infiltrates; 3 = Frequent fungal hyphae with moderate immune infiltrates; 4 = high immune infiltrates with prominent fungal hyphae; 5 = extensive branched filamentous fungal hyphae, immune infiltrates with epithelial damage (21). Fungal burden (CFU/gm of tongue was assessed by incubating the tongue lysates on sabouraud dextrose agar plates for 24 h (28).

Statistical Analyses

P values were calculated by Mann-Whitney test in Prism 6.1 (GraphPad Software, Inc.) assuming random distribution. One and Two way ANOVA analyses were also used for grouped analyses. For correlation, spearman analyses were used. $P < 0.05^*$ was considered significant.

RESULTS

Loss of MyD88 in $Foxp3^+$ Cells Reduces T_{reg} Accumulation in Oral Mucosa *In Vivo*

Although we and others have previously shown that TLR-2/MyD88 signaling can influence mucosal $Foxp3^+$ cells (2, 22, 29, 30), T_{reg} specific role of MyD88 was not evaluated during an infection. To this end, we bred $MyD88^{fl/fl}$ mice with $Foxp3\text{-}YFP^{cre}$ (FYcre) mice and generated MFYcre line in which MyD88 was

deleted in $CD4^+Foxp3^+$ cells (**Figures S1A–C**). Although there was a moderate increase in the frequency of $CD44^{high}$ cells in MFYcre mice, they developed normally without any overt oral inflammation in steady-state conditions (**Figure S2**). We examined the proportions of T_{regs} in spleen (SPLN), oral mucosa draining cervical lymph nodes (CLN), and the mouse oral intra-epithelial lamina propria leukocytes (MOIL) derived from tongue and gingival tissues in these mice. We found that the frequency and absolute numbers of $CD25^+Foxp3^+T_{regs}$ were significantly lower in CLN and MOIL of MFYcre than in control mice (**Figures 1A, B**). However, these were comparable in cells derived from SPLN. Notably, irrespective of the markers used, different T_{reg} subpopulations, namely, $Helios^+$, $Helios^-$, $Nrp1^+$, and ROR-γt^+, were all proportionally reduced (**Figure S3A**). A substantial proportion of T_{regs} was ROR-γt^+ and $Helios^-$ in MOIL, which appeared to be diminished in MFYcre mice (**Figure S3B**). Similar to colonic T_{regs} (13), ROR-γt^+ $Helios^-$ $Foxp3^+$ cells are likely maintained in a microbiome dependent manner in oral mucosa (27, 31). Based on the previous findings on the proliferative effect of *Candida* on T_{regs} (2, 27, 28), we hypothesized that *Candida* may induce local expansion of mucosal T_{regs}, and this expansion might be impaired in MFYcre mice. To test this hypothesis, we treated the cells with heat killed *Candida albicans* germ tube (HKGT) (10^7/ml), α-CD3 (1 μg/ml, α-CD28 (2 μg/ml), and TGF-β1 (5 ng/ml), to examine the frequency of $Foxp3^+$ cells after 5 days. We employed this *in vitro* cell culture system because: 1) We have previously found that HKGT can cause *in vitro* proliferation of T_{regs} in TLR-2 dependent manner; 2) TGF-β1 is important for survival of $Foxp3^+T_{regs}$ during oral CA infection as well resistance to *Candida in vivo* (2, 32, 33); and 3) Activating T cells in a whole tissue culture system including the local antigen presenting cells (APC) is more physiological than using purified T cell cultures because APC secrete appropriate cytokines in the milieu (2, 32). As expected, HKGT stimulation increased the proportion of $Foxp3^+$ cells among $CD4^+$ T cells in FYcre cultures compared to those found *ex vivo* (**Figures 1A, C**, upper panel). However, $CD25^+Foxp3^+$ cells from MFYcre mice expanded much less than FYcre T_{regs} (**Figures 1C, D**). These results show that intrinsic MyD88 signaling in $Foxp3^+T_{regs}$ is a pre-requisite for *Candida* mediated proliferation in TCR activated oral mucosal T_{regs}. To determine the effect of APC and TLR-2 signaling, we sorted $CD4^+CD25^+YFP^+$ cells from FYcre and MFYcre mice and $CD4^+CD25^+$ T_{regs} (> 90% $Foxp3^+$) from TLR-$2^{-/-}$ mice. We then stimulated them with C57BL/6 wild-type (WT) APC. We labelled T_{regs} with cell proliferation dye-670 (CPD670) and compared the proliferation of FYcre, TLR-$2^{-/-}$ and MFYcre T_{regs}. As expected, FYcre T_{regs} proliferated (**Figure 1E**, top 2 panels), but TLR-$2^{-/-}$ showed a moderate reduction in T_{reg} proliferation (**Figures 1E**, 3rd panel). However, MFYcre T_{regs} were significantly more defective in proliferation (**Figures 1E, F**; compare 3rd row with the last row in **Figure 1E**). Therefore, we rationalized that another MyD88 dependent component that is TLR-2 independent should also induce T_{reg} proliferation. Since IL-1R family members signal through MyD88 (34), MFYcre T_{regs} must lack the ability to signal through cytokines such as IL-1β and IL-33 produced by APC and other cells in the milieu. These cytokines have been previously shown to impact mucosal and tissue T_{regs} (35, 36). Therefore, we tested the effects of IL-1β and IL-33 on T_{reg} proliferation. While IL-1β was able to enhance the proliferation of T_{regs} stimulated as above, IL-33 did not (**Figure S4**, top 4 panels). Thus, these results identified a role for intrinsic MyD88/IL-1β signaling in expanding T_{regs} in conjunction with TCR and TLR-2 activation, which could contribute to the compartmentalized regulation of oral mucosal T_{regs}.

Loss of MyD88 in $Foxp3^+$ Cells Exacerbates Oral Inflammation During OPC *In Vivo*

We next determined if MyD88 signaling in T_{regs} alters the physiological outcome of an oral infection and inflammation. As we have previously shown that T_{regs} are crucial for enhancing anti-fungal Th17 cell response and inflammation control at early and later infection phases respectively (27, 28), we hypothesized that MyD88 deficiency in T_{regs} may increase susceptibility to infection and worsen inflammation during OPC. To validate this hypothesis, we sublingually infected MFYcre mice and control mice with CA *in vivo*. On day 14 post infection, we re-infected them to analyze adaptive immune responses. Control mice were infected with PBS control (sham). As a positive control, in one group of MFYcre mice, we intraperitoneally injected 1 x 10^6 $CD4^+CD25^+GFP^+$ T_{regs} from wild-type (WT) Foxp3-GFP reporter mice or $CD4^+CD25^+$ T_{regs} from congenic CD45.1 mice 2 weeks prior to the infection. Seven days after the infection, we assessed the fungal burden in the tongue using Periodic Acid Schiff's (PAS) histochemical staining, which detects fungal hyphae in tongue sections. As anticipated, sham infected control mice did not show fungal presence (**Figure 2A**, **S5A**, 1st and 2nd panels). MFYcre mice showed substantially more hyphae persisting in the tongue compared to FYcre control mice in infected groups (**Figure 2A**, **S5A**, 3rd and 4th panels). MFYcre mice that received adoptively transferred wildtype T_{regs}, however, had fewer hyphae compared to untreated MFYcre mice (**Figure 2A**, **S5A**, 5th panel). Immunohistochemistry showed increased inflammatory infiltrates and reduced numbers of T_{regs} correlating in tongues of MFYcre mice compared to control FYcre mice (**Figure 2B**, left and right, top 2 panels). Determining the fungal growth in tongue lysates also confirmed that reduction of $Foxp3^+$ T_{regs} correlated with increased fungal burden in MFYcre mice (**Figure 2C**, 3rd panel). T_{reg} injected MFYcre mice showed increased infiltrating T_{regs} and concomitant decrease in fungal burden (**Figure 2B**, left and right, bottom panel, **Figure 2C**, three panels). Heightened persistence of inflammatory $F4/80^+Ly6\text{-}C^{high}$ macrophages and $Gr\text{-}1^+$ neutrophils in oral mucosa, even on day 7 after infection, demonstrated tongue immunopathology in infected MFYcre mice (**Figure 2D**, left and right, **S5B, C**). These mice also showed worse weight loss following primary infection and re-infection (**Figure 2E**). Thus, we inferred that the absence of MyD88 in T_{regs} led to an increased fungal burden and continued tissue inflammation, which may trigger a positive feedback loop leading to persistent infection burden.

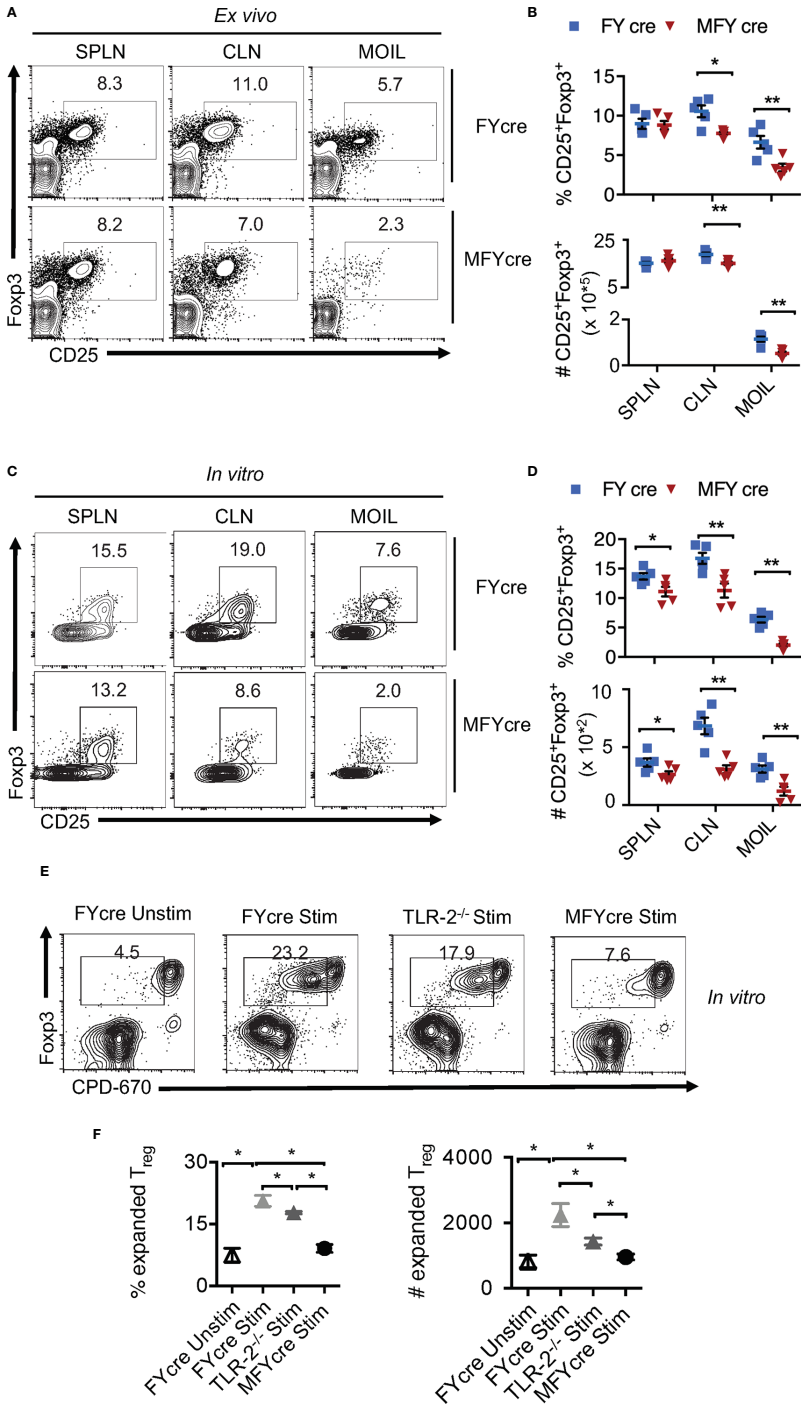

FIGURE 1 | MyD88 deficiency in Foxp3$^+$ cells reduces oral mucosa T_{reg} accumulation *in vivo* and during heat killed Candida albicans germ tube (HKGT) activation *in vitro.* Cells were isolated from spleen (SPLN) and cervical lymph nodes (CLN) and mouse oral intra-epithelial lamina propria leukocytes (MOIL) derived from FYcre and MFYcre mice. **(A)** Flow cytometric plots of CD25 and Foxp3 *ex vivo*. **(B)** Statistics of T_{reg} frequency (above) and numbers (below) from individual mouse from FYcre and MFYcre groups *ex vivo*. **(C)** 3 x 10^5 cells from the indicated tissue were stimulated with α-CD3(1μg/ml, α-CD28 (2μg/ml), TGF-β1 (5 ng/ml) and heat killed *Candida albicans (*CA*)* germ tube (HKGT) (10^7/ml) for 5 days before assessing CD25 and Foxp3 by flow cytometry. **(D)** Statistics of CD25$^+$Foxp3$^+$ cell frequency (above) and numbers (below) in cultures stimulated as in **(C)**, from indicated groups (Each data point corresponds to an individual mouse). **(E, F)** MyD88 signaling expands T_{regs}. CLN CD4$^+$CD25$^+$Foxp3-YFP$^+$ T_{regs} from FYcre and MFYcre mice, and CD4$^+$CD25$^+$ T_{regs} from TLR-$2^{-/-}$ were FACS sorted and labelled with CPD-670. 5 x 10^4 T_{regs} were stimulated with APC as in **C**. Flow cytometric plots showing CPD-670 dilution and Foxp3 **(E)**, and statistics showing T_{reg} expansion **(F)** are depicted. Mean values ± SEM are plotted. (*P < 0.05; Mann Whitney test). Data represent at least triplicate experiments. **P < 0.005.

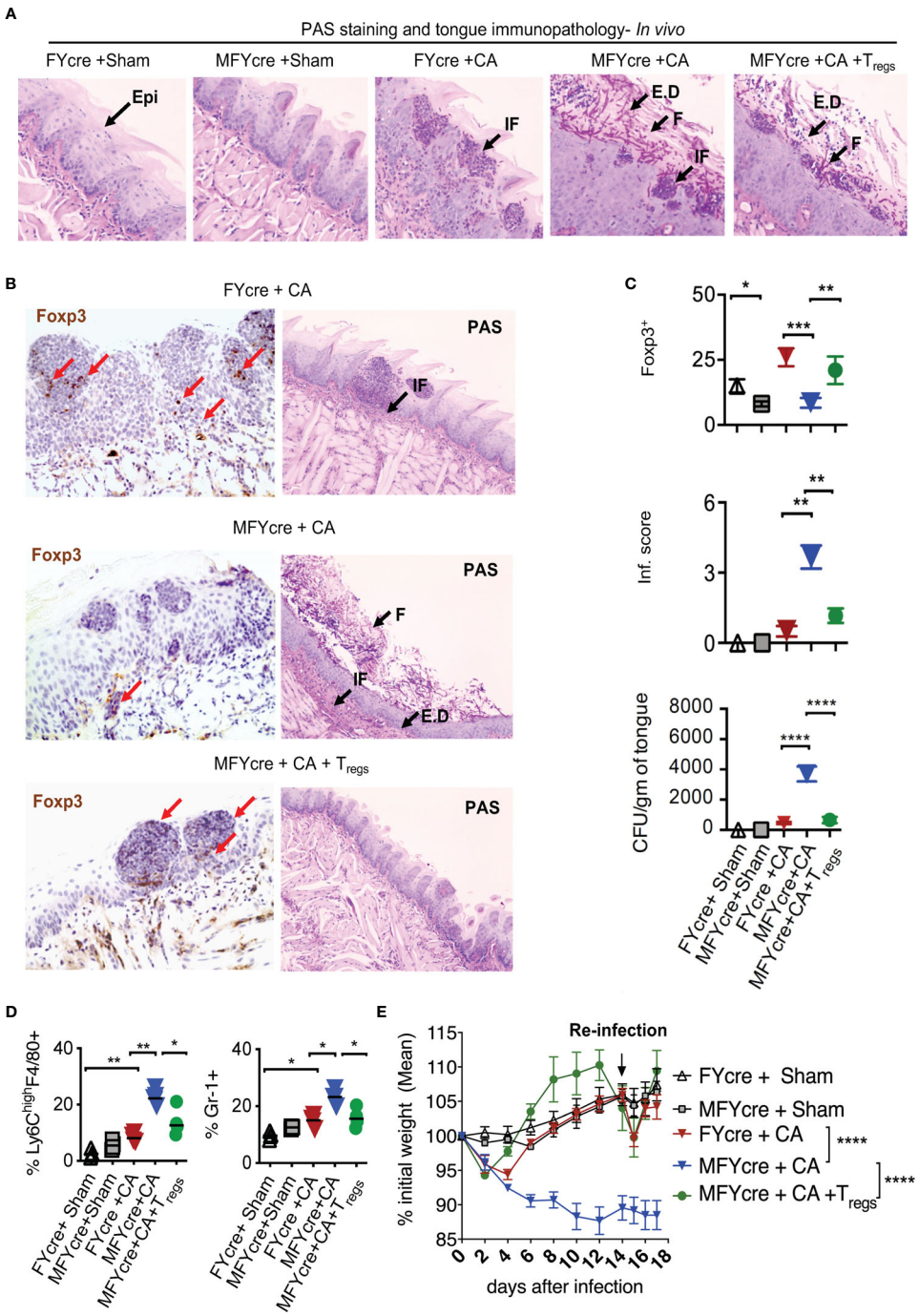

FIGURE 2 | T_{reg} specific deletion of MyD88 reduces results in impaired resistance to oropharyngeal candidiasis (OPC) in mice, and T_{reg} reconstitution reduces fungal burden and immunopathology. FYcre and MFYcre mice were sublingually infected with CA or PBS (Sham) *in vivo* (n= 4-5/group). (MFYcre +CA + T_{regs}) group received 1 x 10^6 $CD4^+CD25^+GFP^+$ T_{regs} from Foxp3-GFP reporter mice. **(A)** Periodic Acid Schiff's (PAS) staining was done on tongue sections isolated from mice on day 7 after infection. **(B)** Foxp3 (left) and PAS (right) immunohistochemistry evaluation was performed on tongues derived from mice on day 7 and 18 after infection, respectively. Microscopic images of the slides viewed at 20X magnification (Epi, epithelium; E.D, epithelial damage; F, fungus; IF, immune cell infiltration; Red arrows indicate some of the $Foxp3^+$cells). **(C)** Statistical analyses of # $Foxp3^+$ (top) cells, inflammation score (middle) from 20X images of the tongues, and fungal burden (CFU/gm of tongue) (bottom panel) assessed in tongue lysates from mice on day 6 or 7 after infection (* P<0.05; Mann Whitney test). **(D)** MOILs were isolated on day-6 after infection and processed for flow cytometric staining of F4/80 and Ly6C (left, **Figure S5B**) and Gr-1 (right, **Figure S5C**). Data represent two experiments. **(E)** Mouse body weight was monitored every other day after infection until the day of sacrifice. The percent weight change in mice in all groups. Mean values ± SEM are plotted. (2 way ANOVA, multiple comparison; alpha= 0.05* significant). At least 3-5 independent experiments showed similar results. **P < 0.005, ***P< 0.0005, ****P < 0.00005.

T_{reg} Specific Deletion of MyD88 Diminishes IL-17A but Increases IFN-γ Expression in Effector Cells and Foxp3$^+$ Cells During Infection

To determine how oral mucosal T_{regs} contribute to immune cell changes during infection, we analyzed the CD4$^+$T cell response in CLN and MOIL in MFYcre mice infected with CA. Examining IL-17A and IFN-γ in Foxp3-negative effector CD4$^+$ T cells on day 3 after re-infection, we found that both sham groups had negligible but comparable levels of cytokine producers. CA infected control mice specifically expressed IL-17A (y-axis) and very little IFN-γ (x- axis) in response to the infection (**Figures 3A-C**). Effector T cells in MFYcre mice, however, produced slightly reduced IL-17A but increased IFN-γ, suggesting a Th1 skewed response when compared to FYcre mice (**Figures 3A, C**). These changes were not observed in SPLN, indicating that immune cell changes were in response to local infection in oral mucosa, as shown previously (27). High proportions of Th17 cells and IFN-γ expressing effector cells (Th*) were observed in MFYcre mice even 26 days after primary infection, indicating a persistent inflammation in these mice. Although there were no differences in IL-10 expression in CD4$^+$Foxp3$^+$ T cell compartment between these groups of mice (**Figure S6A**), MyD88 deficiency in T_{regs} correlates with tongue pathology (**Figure S6B**, **Figure 2**). MFYcre mice that received WT T_{reg} (from congenic CD45.1) injection had significantly lower IFN-γ producing effector cells at all time-points after infection (**Figures 3A, C**, **S6B**, last panel). These results showed that T_{reg}-intrinsic MyD88 signaling is required not only for its proliferation, but also for its ability to control inflammatory IFN-γ producing effector cells. Presence of $T_{reg}17$ cells is known to correlate with the control of immunopathology during OPC (31). While short-chain fatty acids and TLR-2 ligands were involved in promoting $T_{reg}17$ cells through independent mechanisms, T_{reg}-intrinsic MyD88 signaling was not explored (2, 27, 37). Therefore, we examined the proportions of $T_{reg}17$ cells in the oral mucosa, and found that Foxp3$^+$ cells deficient in MyD88 signaling did not show ROR-γt and IL-17A expression during infection (**Figure 3D**). However, CD45.1 WT T_{regs} in MFYcre mice showed ROR-γt and IL-17A expression (**Figures 3D, E**). The proportion of IFN-γ expressing Foxp3$^+$ cells was significantly higher in MFYcre mice than in control mice (**Figures 3F**, 1–4 panels, **G**). Clearly, T_{reg} intrinsic MyD88 signaling was essential for restraining IFN-γ expression in Foxp3$^+$ cells because CD45.1$^+$Foxp3$^+$ cells in MFYcre mice reconstituted with WT T_{regs} expressed lower levels of IFN-γ (**Figures 3F, G**). Previously, IFN-γ expression in T_{regs} has been shown to be associated with human inflammatory diseases and dysfunction in T_{reg} cells (38). Our results are also consistent with this notion, because T_{reg}IFN-γ appeared to be dysfunctional (T_{regDys}) and positively correlated with increased immunopathology in infected mice (**Figures 2**, **3H**). In contrast, increased $T_{reg}17$ cells positively correlated with lower inflammation score in infected mice (**Figure 3H**). These results show that T_{reg}-intrinsic MyD88 signaling is critical in controlling their functions by differentially modulating their expression of IL-17A and IFN-γ. Defects in this signaling converts host defense Th17 response in to an immunopathological response during oral *Candida* mucosal infection.

Loss of MyD88 in T_{regs} Abrogates Their Suppressive Activity *In Vitro*

The above-mentioned results suggest that the absence of MyD88 in Foxp3$^+$$T_{regs}$ not only impair early Th17 responses, but also render T_{regs} unable to control excessive CD4$^+$T cell responses during the resolution phase of infection. To further verify this possibility, we determined the ability of FYcre and MFYcre T_{regs} isolated from infected mice to suppress CD4$^+$T cells *in vitro*. To this end, we isolated CLN and MOIL from infected mice at a late phase of infection and re-stimulated them in the presence or absence of T_{regs} in cultures. For removal of T_{regs} in cultures, we depleted CD4$^+$CD25$^+$ cells before re-stimulation with α-CD3 and α-CD28 antibodies. T_{reg} depletion led to a decrease of CD25$^+$Foxp3$^+$ cells from 24.8% to 2.1%, 9.2% to 1.4%, and 18.6% to 2.3% in FYcre, MFYcre, and MFYcre + WT T_{reg} cultures respectively (data not shown). We then examined the proliferation of Foxp3 negative effector CD4$^+$ T cells by 5-bromo-2'-deoxyuridine (BrdU) labeling assay. The cells with no re-stimulation did not undergo proliferation (Unstim, **Figures 4A**, top row, B). As expected, depletion of FYcre T_{regs}, but not MFYcre T_{regs} *in vitro*, significantly increased the proliferation of responding CD4$^+$ T cells in their respective cultures (**Figures 4A**, 1st 2 columns, **B**). More importantly, WT T_{regs} that were injected *in vivo* also retained their suppressive capacity *in vitro* and inhibited the proliferation of MFYcre CD4$^+$ T cells (**Figures 4A**, 3rd column, **B**). We and others have previously shown that T_{regs} can downmodulate the sensitivity of effector cells to IL-2 by reducing their IL-2 receptor (CD25) expression (26, 28, 39). Therefore, we determined the expression of CD25 on Foxp3^{negative} effector cells in these cultures. As expected T_{regs} downmodulated CD25 expression in FYcre cultures and MFYcre cultures with WT T_{regs} (**Figures 4C**, 1st and 3rd columns, **D**). However, depletion of MFYcre T_{regs} did not affect CD25 expression in effector cells (**Figures 4C**, 2nd column, **D**), suggesting that MFYcre T_{regs} had impaired ability to control CD25 expression and excessive CD4 T cell responses. Collectively, these data confirm a nonredundant role of MyD88 in immunomodulatory function of Foxp3$^+$ cells during the later phase of the infection *in vivo*.

Induction and Proliferation of $T_{reg}17$ and T_{regDys} Cells Are Driven by IL-1β and IL-6, Respectively

Based on our *in vivo* experiments that showed MyD88's role in promoting $T_{reg}17$ cells and inhibiting T_{regDys} cells, we then examined how MyD88 deficiency affects T_{regs} during *Candida* stimulation *in vitro*. Therefore, we stimulated CLN and MOIL cells from FYcre and MFYcre mice with HKGT as in **Figure 1C**, and determined $T_{reg}17$ induction. As a control, we also stimulated purified TLR-2$^{-/-}$ T_{regs} with HKGT and wildtype APC. Although HKGT mediated $T_{reg}17$ induction was unaffected in TLR-2$^{-/-}$ T_{regs}, it was significantly reduced in T_{regs} lacking MyD88 *in vitro* (**Figure 5A**, left and right). This indicated that HKGT mediated $T_{reg}17$

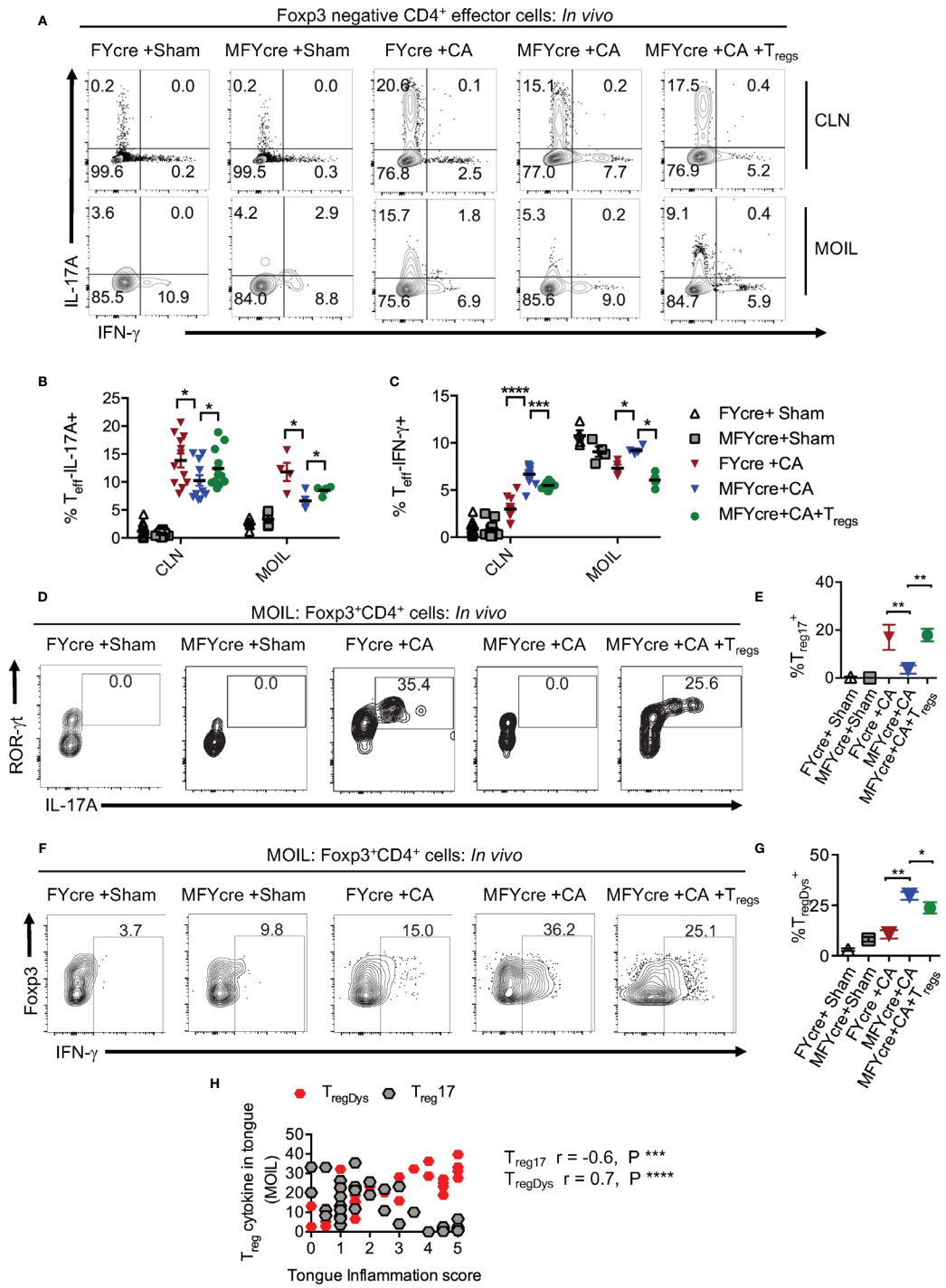

FIGURE 3 | Loss of MyD88 in T_{regs} diminishes IL-17A and increases IFN- γ expression in effector cells and Foxp3$^+$ cells *in vivo* during oropharyngeal candidiasis (OPC) infection. FYcre and MFYcre mice were infected with sham control or CA as in **Figure 2** (n= 4–6/group). On day 3 after infection, cells from cervical lymph nodes (CLN) and mouse oral intra-epithelial and lamina propria leukocytes (MOIL) were restimulated with PMA-ionomycin to assess intracellular proteins. MOIL cells pooled from 2 mice were used for flow cytometric analyses. **(A)** Flow plots of IL-17A and IFN-γ, gated on CD3$^+$CD4$^+$ Foxp3$^-$ effector (T_{eff}) cells. Statistical analyses of T_{eff} IL-17A$^+$ **(B)**, and T_{eff} IFN-γ$^+$ **(C)**. Flow plots of ROR-γt and IL-17A **(D)**, Foxp3 and IFN-γ, **(F)** gated on CD3$^+$CD4$^+$ Foxp3$^+$$T_{reg}$ cells, and statistical analyses of the proportion of ROR-γt$^+$IL-17A$^+$ T_{regs} (% T_{reg17}) **(E)**, and T_{reg} IFN-γ$^+$ (% T_{regDys}) **(G)**, in different groups are shown. For statistical analyses, data are pooled from two experiments. Each data point represents one mouse in CLN and 2 mice in MOIL. Mean values ± SEM are plotted. **(H)** Correlation curve was plotted using values from tongue inflammation score, %MOIL $T_{reg}17$ and %MOIL T_{regDys} in infected mouse groups. These data represent three independent experiments showing similar results. *P < 0.05, ***P < 0.005, ***P < 0.0005, ****P < 0.00005.

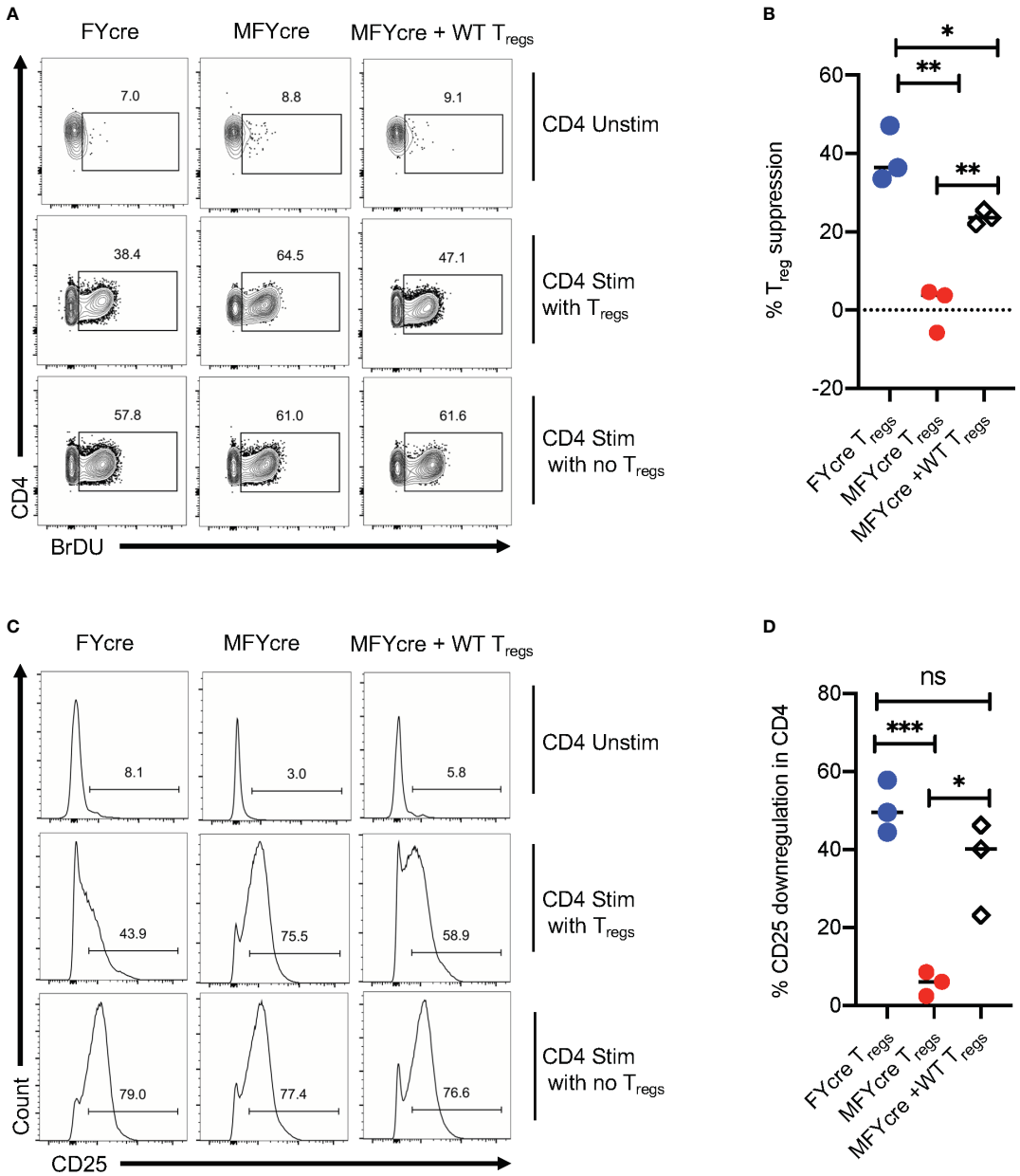

FIGURE 4 | $CD25^+Foxp3^+$ cells lacking MyD88 in infected mice lack suppressive functions *in vitro*. FYcre and MFYcre mice were infected with sham control or CA as in **Figure 2** (n= 4–6/group). On day 6 after infection, cells from cervical lymph nodes (CLN) and mouse oral intra-epithelial and lamina propria leukocytes (MOIL) of FYcre + CA, MFYcre + CA and MFYcre + CA +T_{regs} groups were collected for *in vitro* stimulation with α-CD3 and α-CD28 antibodies (CD4 Stim with T_{regs}) for 3–4 days. Some cells each mouse group were stimulated after the removal of $CD4^+CD25^+$ T_{regs} (CD4 Stim with no T_{regs}). A control culture was plated with no restimulation (CD4 Unstim). BrDU was added in the last 24 h before harvesting the cells for fixation and flow cytometry for BrDU **(A, B)** and CD25 **(C, D)**. Flow cytometry gating excluded $Foxp3^+$ T_{reg} cells in cultures. T_{reg} suppression was measured by % BrDU+ proliferating cells as normalized to "CD4 Stim with no T_{regs}" cultures. Mean values ± SEM from 3 independent experiments are plotted. * P< 0.05; 2 way ANOVA and Unpaired 2-tailed students "t" tests. **P < 0.005, ***P < 0.0005. NS, non-significant.

induction is dependent on MyD88 expression and not TLR-2 expression in T_{regs}, suggesting the involvement of IL-1/MyD88 signaling. Because we observed that IL-1β signaling is involved in *Candida* dependent T_{reg} proliferation (**Figure S4**) and Th17 cell induction in mucosa (40), we focused on IL-1β, which we propose to be secreted by APC in the milieu. We stimulated CLN/MOIL cells with TCR activating antibodies along with TGF-β1, HKGT, and IL-1β, alone or in combination (**Figure S7**). We analyzed Foxp3 (y-axis) and IL-17A (x-axis) expression in $CD4^+$ T cells on day 5 after stimulation. While HKGT increases the frequency of $Foxp3^+$ cells and $T_{reg}17$ cells, IL-1β, and TGF-β1 alone did not induce $T_{reg}17$ cells. However, a combination of these reagents synergistically promoted an increase in the frequency of $T_{reg}17$ cells (**Figure S7**). While adding exogenous IL-1β increased the

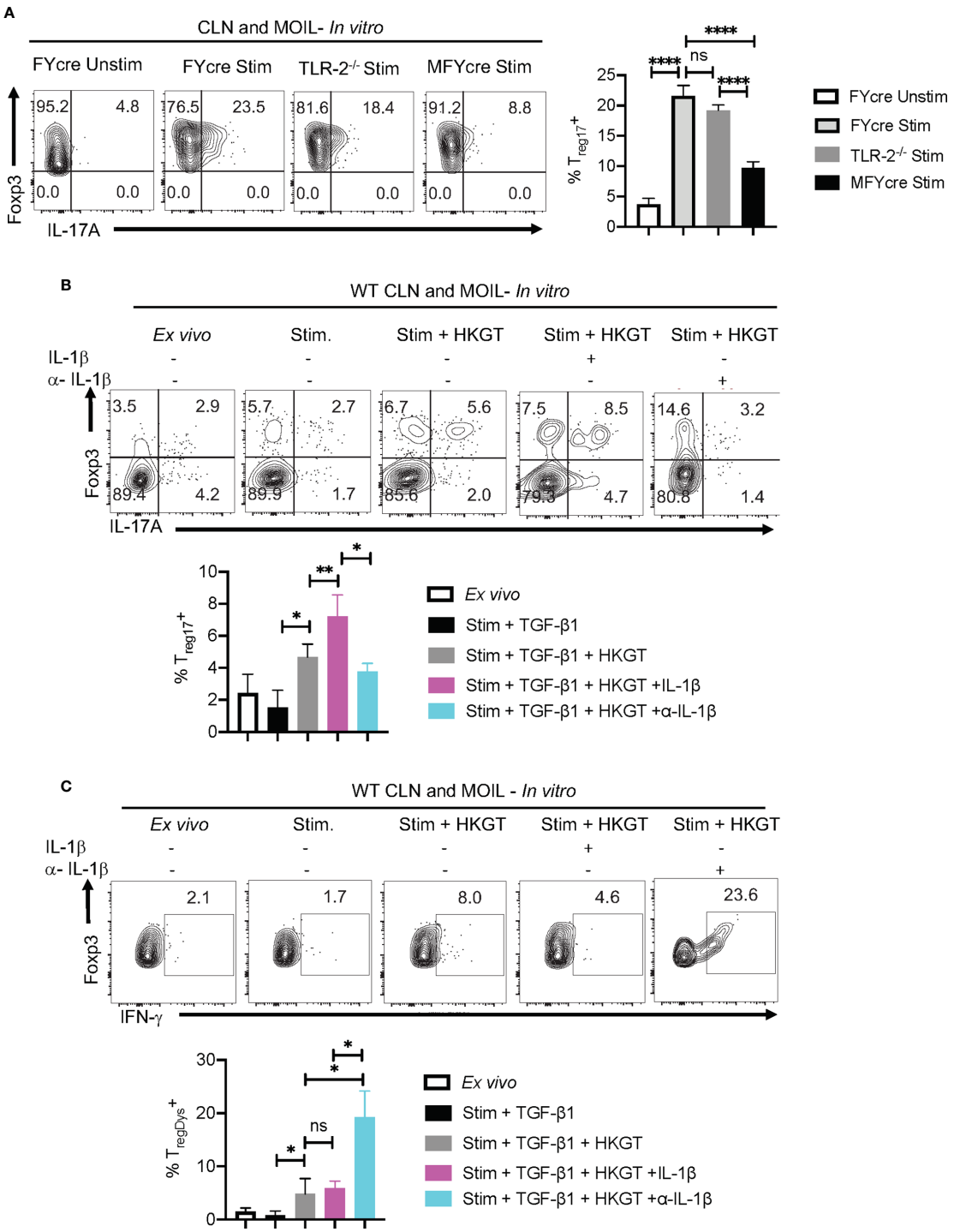

FIGURE 5 | IL-1β promotes $T_{reg}17$ cells and constrains induction of T_{regDys} cells in *Candida* activated oral mucosal cells *in vitro*. **(A)** HKGT mediated $T_{reg}17$ induction is slightly reduced in TLR-$2^{-/-}$ T_{regs}, but significantly lower in MFYcre T_{regs} *in vitro*. Pooled cervical lymph nodes (CLN) and mouse oral intra-epithelial and lamina propria leukocytes (MOIL) cells from FYcre and MFYcre were stimulated with heat killed *Candida albicans* germ tube (HKGT) as in **Figure 1C** for 5 days and were restimulated with PMA-Ionomycin for flow cytometry assessment. For TLR-$2^{-/-}$ cultures, T_{regs} were purified from TLR-$2^{-/-}$ mice and stimulated with WT APC from T cell depleted CLN and MOIL cells. Flow cytometry plots showing Foxp3 and IL-17A expression (gated on $CD3^+CD4^+Foxp3^+$ cells). **(B, C)** WT CLN and MOIL cells were examined *ex vivo* or stimulated with HKGT as in **Figure 1C**, in the presence of IL-1β (10 ng/ml) or α- IL-β antibody (10 μg/ml) for 5 days. Flow cytometry plots showing Foxp3 and IL-17A expression (gated on $CD3^+CD4^+$ cells) (**B**, top) and statistical data points from experimental replicates (**B**, bottom). Flow plots showing Foxp3 and IFN-γ expression (gated on $CD3^+CD4^+Foxp3^+$ cells) **(C)** and statistical data points from experimental replicates (**C**, bottom). NS, non-significant. *P < 0.005, **P < 0.0005, ****P < 0.00005.

frequency of $T_{reg}17$ cells, blocking IL-1β significantly diminished $T_{reg}17$ cells (**Figure 5B**, top and bottom).This result suggested that APC produce endogenous IL-1β. As expected, IL-1β dependent IL-17A expression was also observed in Foxp3-negative cell compartments (**Figure 5B**). Surprisingly, while exogenous IL-1β decreased IFN-γ expression in $Foxp3^+$ cells, antibody mediated blocking of endogenous IL-1β heightened the frequency of IFN-γ expressing $Foxp3^+$ cells (**Figure 5C**). To further evaluate the involvement of IL-1 receptor signaling in induction and proliferation of $Foxp3^+T_{regs}$, we stimulated naïve $CD4^+$ T cells from WT C57BL/6 and IL-1R knockout mice ($IL\text{-}1R1^{-/-}$) with HKGT along with APC from CLN. HKGT promoted $T_{reg}17$ cells in IL-1β/TGF-β1 stimulated WT cells but not in $IL\text{-}1R1^{-/-}$ cells (**Figure 6A**). These results demonstrated the role of endogenous IL-1β in promoting IL-17A expression in induced $Foxp3^+$ cells (**Figure 6A**, upper right quadrants, top, and bottom). Although the effects were moderate, the frequency of IFN-γ was consistently higher in $IL\text{-}1R1^{-/-}$ $CD4^+$ T cells than in WT cells (**Figure 6B**, upper right quadrants, top, and bottom). Corroborating these flow cytometry data, quantitative PCR (qPCR) of $IL\text{-}1R1^{-/-}$ $CD4^+$ T cells from these cultures showed relatively lower abundance of ROR-γt and IL-17A mRNA, but slightly higher levels of Foxp3 and IFN-γ mRNA than WT $CD4^+$ T cells (**Figure 6C**). To validate IL-1β effects *in vivo*, we orally infected WT and $IL\text{-}1R1^{-/-}$ mice with CA and examined $Foxp3^+$ cells in MOIL three days after infection and *in vitro* restimulation. While T_{reg} proportions were comparable in uninfected mice (**Figure S8**), infected $IL\text{-}1R1^{-/-}$ mice displayed strikingly lower frequencies of $T_{reg}17$ cells and higher T_{regDys} cell proportions compared to WT cells *in vivo* (**Figures 6D, E**). Because IL-6 is also required for Th17 cell differentiation, we determined the role of IL-6 in IL-17A expression in T_{regs}. Surprisingly, blocking IL-6 using an antibody reduced $T_{reg}17$ proportions only in WT, but not in $IL\text{-}1R1^{-/-}$ cultures. However, it significantly diminished the frequency of T_{regDys} in $IL\text{-}1R1^{-/-}$ cells (**Figures 6D, E**). Although mechanisms of contrasting effects of IL-6 and IL-1β are unclear at the molecular level, our data show that IL-1β is key in generating $T_{reg}17$ cells and inhibiting IL-6 dependent IFN-γ expression in $Foxp3^+$ cells during *Candida* stimulation. Collectively, the data presented herein confirm the expected role of IL-1β and IL-6 signaling in $CD4^+$ T cells, but they also reveal new roles for these signaling pathways in regulating T_{reg} functional plasticity.

IL-1β Induced T_{reg17} Cells Requires m-TOR Activation

Considering the direct effect of IL-1β/MyD88 axis on $CD4^+$ T cells in inducing $T_{reg}17$ cells, we next determined the biochemical basis underlying IL-1β signaling in purified naïve cells and T_{regs} *in vitro*. We labeled the cells with carboxyfluorescein diacetate succinimidyl ester (CFSE), added 1-10 ng/ml of IL-1β in some cultures at the beginning of stimulation, and assessed them after 3 days. First, we analyzed IL-1R-associated kinase 4 (IRAK) phosphorylation (p-IRAK4) as a surrogate for MyD88 signaling, and found that T_{reg} cells had slightly higher levels of p-IRAK-4 expression with HKGT alone when compared to naive cells (**Figure 7A**). This result is consistent with an observed TLR-2 expression on T_{regs} (2). The addition of IL-1β further increased p-IRAK4 expression, both in naïve and T_{reg} cells in a concentration dependent manner (**Figure 7A**). Since IL-1β has been previously implicated in NF-κB activation in immune cells (41), we examined whether it impacts activation and nuclear translocation of NF-κB in HKGT stimulated T cells. Confocal microscopic analyses revealed that IL-1β did not promote NF-κB translocation in activated naïve and T_{reg} cells (**Figure S9**). Because IL-1β can affect Akt/mTOR metabolic signaling proteins in T cells (42, 43), we turned to examining phosphorylated-Akt (p-Akt) and phosphorylated-mammalian target of Rapamycin (p-mTOR), which are the activated forms of these proteins. IL-1β was able to increase p-Akt and p-mTOR expression in naïve and $Foxp3^+$ $CD4^+$ T cells in a concentration dependent manner (**Figures 7B, C**). We then determined the levels of a target protein of mTOR, namely phosphorylated-70-S6K (p-70-S6K), by confocal analyses of CFSE labeled T_{regs}. While TCR and HKGT stimulation alone caused CFSE dilution (**Figure 7D**, first two panels), IL-1β further enhanced p-70-S6K in proliferating $Foxp3^+$ cells in a concentration dependent manner (**Figure 7D**, last two panels). To evaluate these IL-1β mediated effects *in vivo*, we analyzed the expression of mTOR in $Foxp3^+$ cells in oral mucosa (MOIL) derived *ex vivo* and from *Candida* infected WT and $IL\text{-}1R1^{-/-}$ mice. *Candida* infection promoted mTOR phospohorylation in WT but not in $IL\text{-}1R1^{-/-}$ T_{regs} *in vivo* (**Figure 7E**). Akt/mTOR signaling is well established in T_{reg} and Th17 homeostasis and functions in other settings (31, 44–46). Therefore, we hypothesized that IL-1β signaling might enhance IL-17A in $Foxp3^+$ cells in an mTOR dependent manner in the context of *Candida* activation. To this end, we determined IL-17A in HKGT/IL-1β stimulated CLN/MOIL cells in the presence and absence of an mTOR inhibitor Rapamycin (Rapa) *in vitro*. Gating on $Foxp3^+$ cells in these cultures revealed that HKGT/IL-1β dependent $T_{reg}17$ induction was blunted in Rapa treated cells (**Figure 7F**, left and right). Collectively, these results showed that IL-1β/IL-1R signaling is required for promoting mTOR activation and IL-17A induction in $Foxp3^+$ cells during *Candida* infection in oral mucosa.

Candida Infected Aged Mice Display Immunopathology, Lower IL-1β Induction, but Excessive Secretion of IL-6

Our results imply that *Candida* infection in the context of IL-1β/IL-6 imbalance may lead to an inappropriate accrual of dysfunctional $IFN\text{-}\gamma^+Foxp3^+$ cells. Interestingly along these lines, aging is associated with elevated levels of serum IL-6 and $IFN\text{-}\gamma^+$cells, increased prevalence of $Foxp3^+$cells in blood, immune system decline (immunosenescence), and an exaggerated inflammatory state (inflammaging) (47–53). Therefore, we next investigated these cytokines in the context of MyD88/IL-1β signaling and T_{reg} functions during *Candida* infection in aged mice. To this end, we sublingually infected young (6-8 weeks of age) and aged (12–18 months of age) mice with *Candida* and monitored their immunopathology, weight loss and fungal burden. Examining the inflammatory infiltrates on day 20 after initial infection showed there was increased immunopathology in infected aged mice when compared to younger counterparts (**Figure 8A**). Infection caused significantly more weight loss

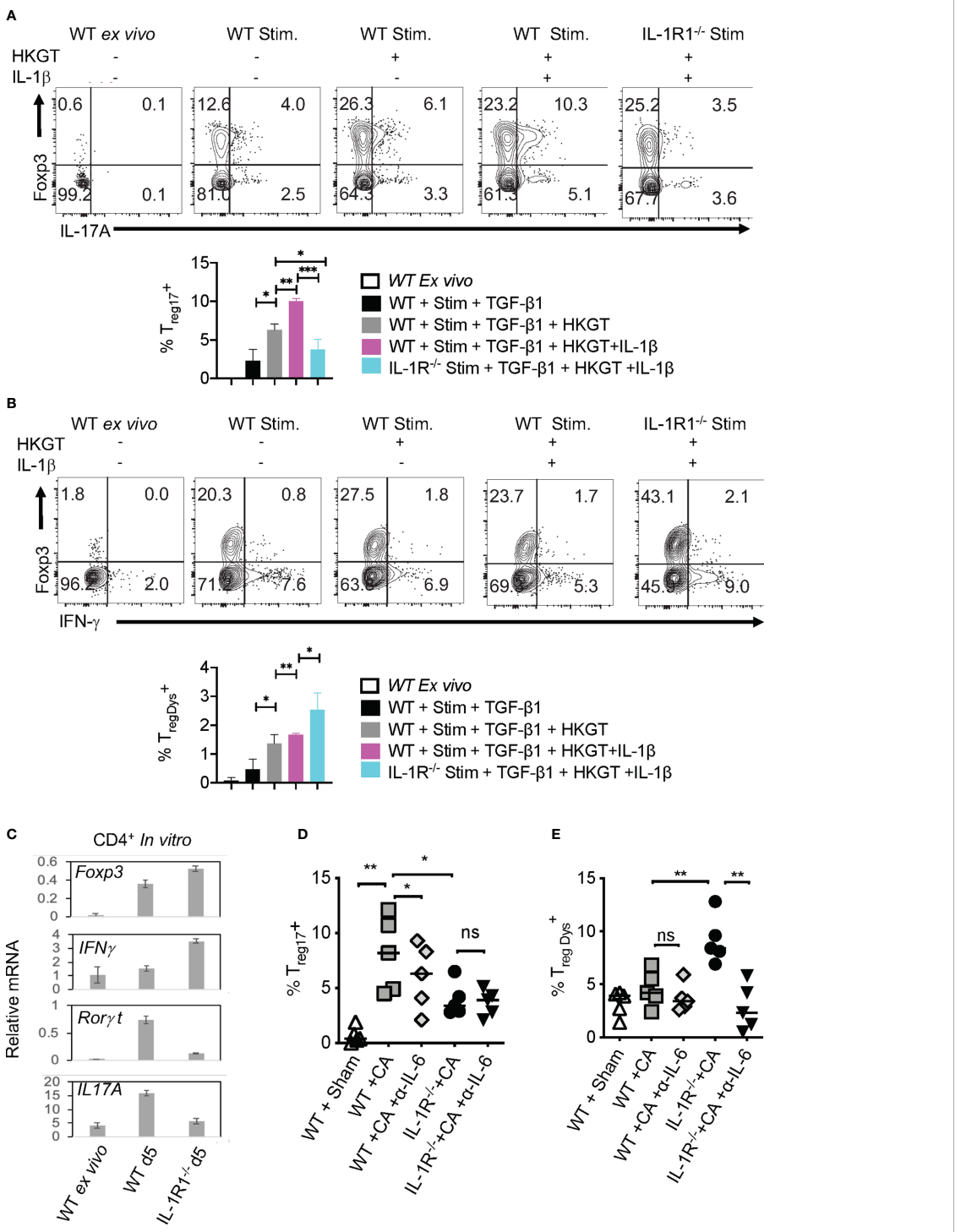

FIGURE 6 | Loss of endogenous IL-1R signaling diminishes $T_{reg}17$ but increases T_{regDys} cells in $CD4^+$ T cells *in vitro*. IL-6 expands T_{regDys} cells in the absence of IL-1β. Naïve $CD4^+$ T cells from WT C57BL/6 and IL-1R ($IL\text{-}1R1^{-/-}$) knockout mice were stimulated with heat killed *Candida albicans* germ tube (HKGT) and TGF-β1 for 5 days as in **Figure 1C** with WT APC and stained for Foxp3, IL-17A (**A**, top and bottom), and Foxp3 and IFN-γ (**B**, top and bottom) (gated on $CD3^+CD4^+$ cells). **(C)** Naïve $CD4^+$ T cells from WT C57BL/6 and IL-1R ($IL\text{-}1R1^{-/-}$) knockout mice were stimulated with HKGT and TGF-β1 for 3 days. CD4 T cells were purified from these cultures for qPCR assessment of indicated transcripts. **(D, E)** WT and $IL\text{-}1R1^{-/-}$ mice were orally infected with CA, and cervical lymph nodes (CLN) and mouse oral intra-epithelial and lamina propria leukocytes (MOIL) were collected 3 days after infection (n=5/group) for HKGT restimulation for 2 days and flow cytometry. Statistical analyses of $Foxp3^+ROR\text{-}\gamma t^+IL\text{-}17A^+$ **(D)**, and $Foxp3^+$ $IFN\text{-}\gamma^+$ **(E)**, expressing cells after PMA/Iono restimulation for 4 h *in vitro* (gated on $CD3^+CD4^+$ cells). α- IL-6 antibody (10 μg/ml) was added in some cultures. Data are representative of at least 3 independent experiments. NS, non-significant. *P < 0.05, **P < 0.005, ***P < 0.0005.

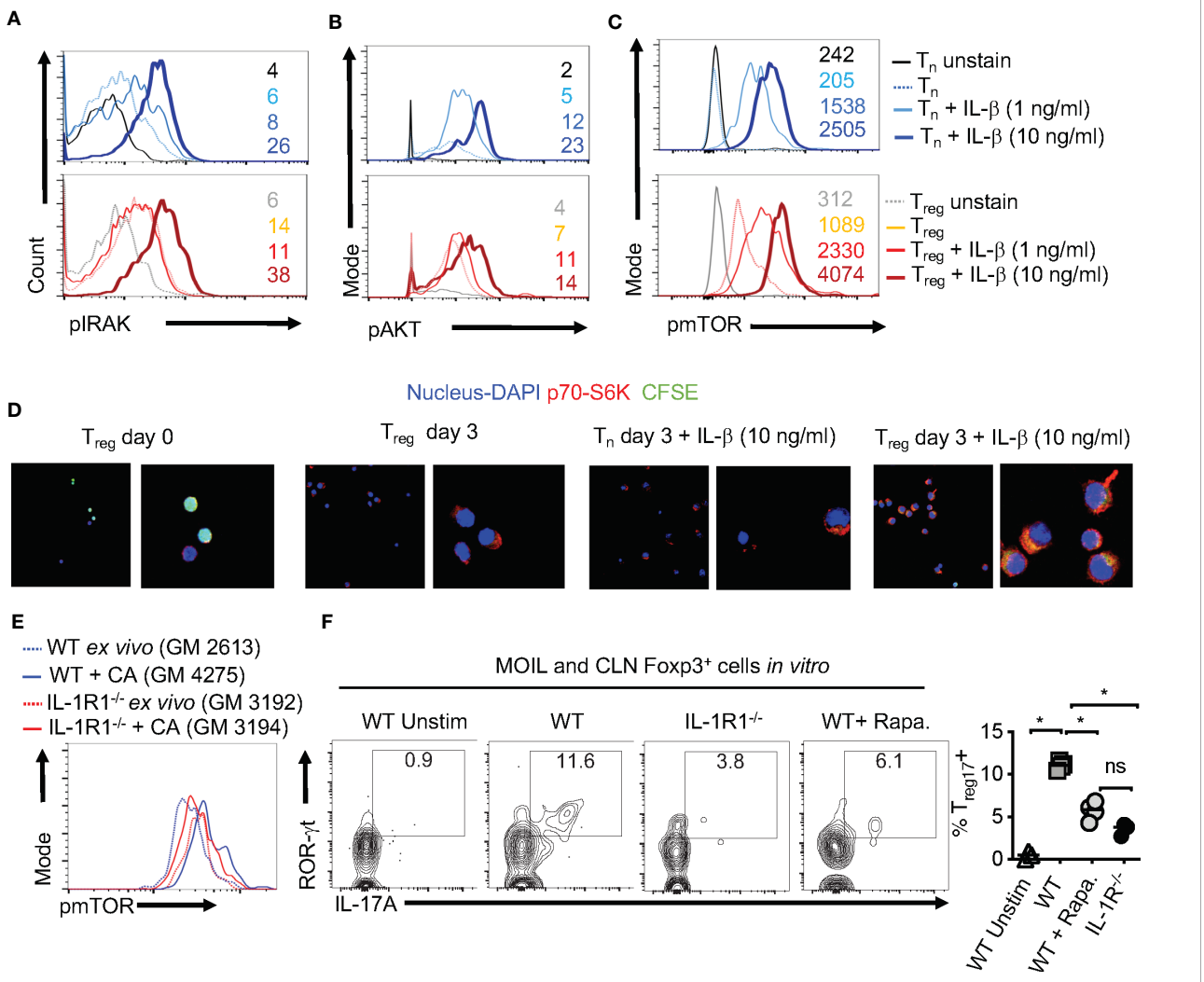

FIGURE 7 | IL-1β induces IRAK, Akt, TOR, and p-70-S6K activation in T_{regs} **(A)** Naïve $CD4^+$ T (T_n) and $CD4^+CD25^+T_{reg}$ cells were CFSE labeled and stimulated as in **Figure 1C** for 3 days without or with IL-1β. Cells were restimulated with 10 ng/ml of IL-1β for 4 h before fixation and staining for p-IRAK **(A)**, p-Akt **(B)**, p-mTOR **(C)**, and p-70-S6K **(D)** for flow cytometry analyses, or cytospun on slides for confocal analyses. **(E)** Mouse oral intra-epithelial and lamina propria leukocytes (MOILs) were collected from oral CA infected WT and IL-$1R1^{-/-}$ mice 3 days after infection (n=5/group) and stained for p-mTOR as in **(C)**. (**F**, left and right) Pooled cervical lymph nodes (CLN) and MOIL cells were stimulated with HKGT as in **Figure 1C** for 5 days with or without Rapamycin (1 ng/ml Rapa). Cells were restimulated with PMA/Ionomycin before intracellular staining and flow cytometry analyses (gated on $Foxp3^+$ cells). Geometric mean fluorescence intensities (GM) are shown with the histogram plots. Data represent triplicate experiments with similar results. NS, non-significant. *P < 0.05.

(**Figure 8B**, top) and persistence of infiltrating inflammatory cells (Inf. score) in aged mice than in young mice (**Figure 8B**, bottom). Remarkably, PAS histochemical staining and culturing tongue lysates to determine the fungal growth revealed that there was no significant increase in fungal burden in aged tongues (**Figure 8C**). These results indicated that both young and aged mice were capable of clearing the fungal infection in a similar manner (**Figure 8C**). We then analyzed the $CD4^+$ T cells in oral mucosal tongue tissues. The basal levels of $Foxp3^+$ cells were proportionally higher in SPLN, CLN and MOIL in aged uninfected mice (**Figures 8D**, **S10**). However, we observed blunted expression of CD25 in aged $Foxp3^+$ cells (**Figure 8D**, X axis; see legend). Upon OPC infection, we found that both the frequency and number of $Foxp3^+$ cells increased in both the groups, but at significantly higher levels in aged mice than in young mice (**Figures 8D**, **S10**). Finally, to evaluate the role of IL-1β and IL-6 imbalance in aged mice, we examined the levels of these cytokines along with other pro-inflammatory cytokines using Enzyme-linked immunosorbent assay (ELISA). ELISA of oral tongue tissues (MOIL) 2 days after infection showed that while infection promoted the expression of IL-1β in MOIL of young mice, cells derived from aged MOIL produced little or no IL-1β (**Figure 8E**, top panel). This result was observed in MOIL and CLN, but not in SPLN, again highlighting a compartmentalized and infection- mediated effects in T cells of oral

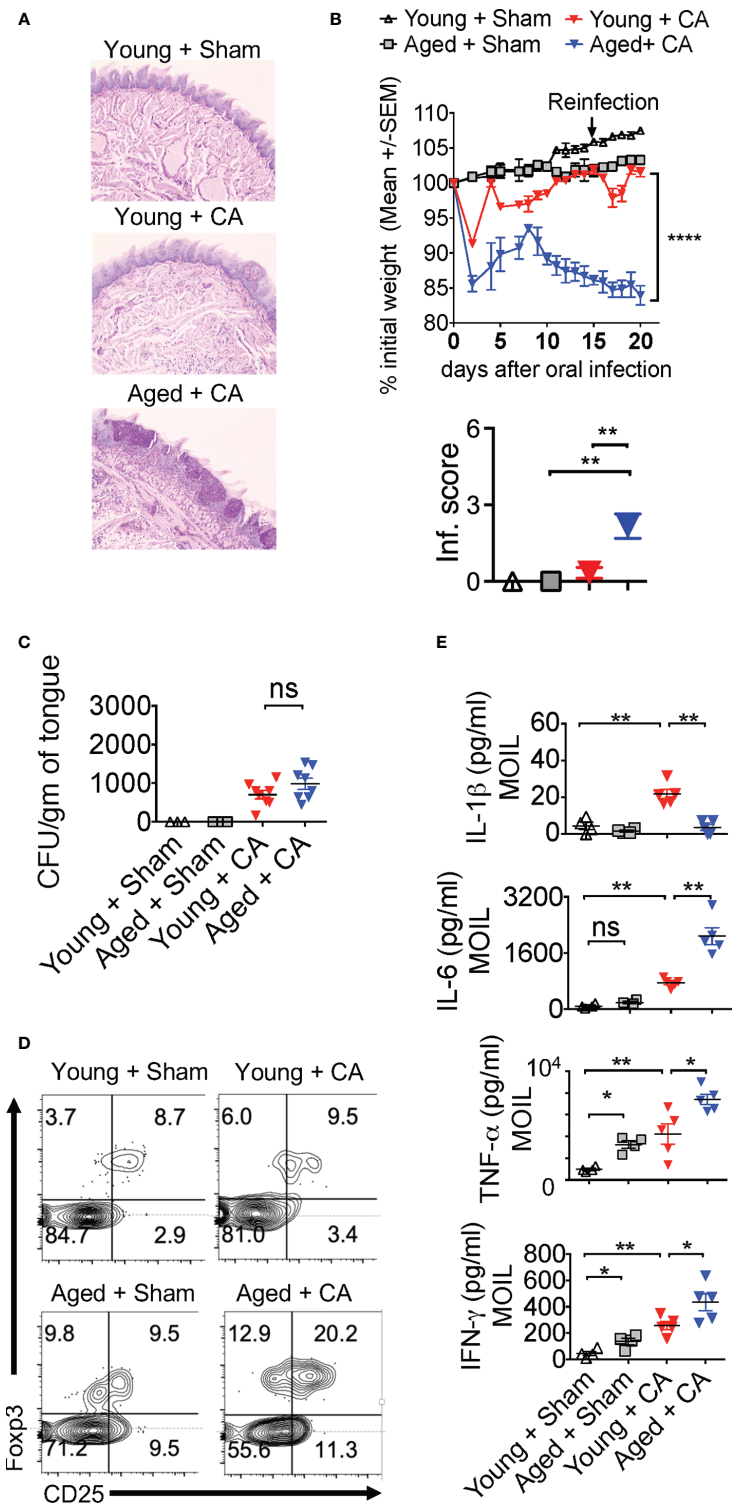

FIGURE 8 | Aged mice show diminished IL-1β but excessive IL-6 expression and immunopathology during OPC. Young (6 weeks) and Aged (age 12–18 months) mice were sublingually infected and re-infected with CA or Sham (n= 4–5/group) as in **Figures 2** and **3**. Histological analyses of PAS staining **(A)**, body weight (**B** above), tongue inflammation score (**B**, below) and fungal burden in tongue lysates **(C)** on day 7 of infection are shown. **(D)** Flow cytometric analyses of oral mucosal $CD3^{+}CD4^{+}$ gated cells for CD25 and Foxp3; Geometric means of CD25 in Foxp3+ cells: Young + Sham= 776; Young +CA= 851; Aged + Sham= 334; Aged + CA=618. **(E)** Supernatants were collected from MOIL cells restimulated with PMA/Ionomycin, and IL-1β (on day 2 after infection), and IFN-γ, IL-6, and TNF-α levels (day 5 after infection) were quantified by ELISA. NS, non-significant. *P < 0.005, **P < 0.0005, ****P < 0.00005.

mucosa (**Figure S11A**). However, concurrent to increased immunopathology in aged mice, aged MOIL supernatants revealed highly elevated levels of pro-inflammatory cytokines such as IL-6, TNF- α, and IFN-γ, substantiating the presence of oral immune hyperactivation in aged mice (**Figure 8E** rows 2–4, **S11B**). Collectively, these results show that tongue immunopathology could be ascribed to an impairment of IL-1β and excessive production of IL-6 during oral *Candida* infection in aged mice.

Aged Mice Display Dysfunction in Foxp3$^+$ Cells During *Candida* Infection

Despite increased proportions of Foxp3$^+$ cells, loss of IL-1β may render aged Foxp3$^+$ cells dysfunctional, similar to MyD88$^{-/-}$ T_{regs} (**Figures 2–4**). This may provide a positive feedback inflammatory loop to further exacerbate immunopathology during infection. To validate this hypothesis, we analyzed T_{reg} cytokine expression using flow cytometry. Remarkably, the proportion of $T_{reg}17$ cells was significantly lower in CLN and MOIL in aged mice compared to young mice (**Figures 9A**, upper right quadrants, **B**). There were no significant differences between the young and aged groups in IL-17A production by non T_{regs} (Foxp3 negative; Th17) (**Figures 9A**, lower right quadrants, **B**). Foxp3$^+$ cells from young mice produced very little IFN-γ, while those derived from aged mice displayed higher levels of IFN-γ production (**Figures 9C**, upper right quadrants, **D**). This effect was much more pronounced in oral mucosa of infected aged mice, reminiscent to MFYcre T_{regs} *in vivo* (**Figure 3**). Both Foxp3$^+$ cells and IFN-γ$^+$ effector cells were present at higher proportions in aged mice, suggesting an impairment in immunomodulatory functions in these Foxp3$^+$ cells (**Figures 9C**, lower right quadrant, **D**). More importantly, aged infected mice accrued pro-inflammatory effector CD4$^+$ T cells expressing IFN-γ and IL-17A in oral mucosa even at later time points (day 30 after initial infection), indicating that the infection had led to the persistence of inflammatory CD4$^+$ cells despite the absence of fungal burden in these mice (**Figures 8C**, **S11C**). To address the IL-1β/MyD88 mediated effects in Foxp3$^+$ cells *in vivo*, we analyzed the expression of p-IRAK4 and IL-1R1 in Foxp3$^+$ cells in oral mucosa (MOIL) derived from infected mice. Corroborating with lower IL-1β levels in aged mice (**Figure 8E**, top panel), Foxp3$^+$ cells showed lower p-IRAK phosphorylation when compared to young mice (**Figure 9E**, left and right). These data suggest that IL-1β expressed during early time-points of infection may phosphorylate p-IRAK4 in IL-1R$^+$$T_{regs}$, which is critical for the activation and proliferation of these cells in oral mucosa. Furthermore, Foxp3$^+$ cells from aged mice displayed lower levels of mTOR phosphorylation upon infection when compared to young mice (**Figure 9F**, top and bottom). In summary, these results indicate that excessive immunopathology in aged mice may be attributed to lower mTOR phosphorylation in Foxp3$^+$ cells and diminished $T_{reg}17$ cells during oral infection. These $T_{reg}17$ defects also paralleled with impaired IL-1β induction, while excessive T_{regDys} accumulation could be attributed to elevated levels of IL-6 in oral mucosa of aged mice. These data are consistent with our *in vitro* results showing the contrasting roles of IL-6 and IL-1β on T_{reg} functions.

Human Oral Mucosal Immune Dysfunction in Aged Individuals Parallels T_{reg} Alterations

Human aging is associated with systemic inflammation and oral manifestations associated with elevated IL-6 levels and immune dysfunction (54–56), although the underlying defects in immunomodulatory mechanisms are unclear. Based on the above results in aged mice, we turned to interrogate the physiological relevance of IL-1β/MyD88 defects and T_{reg} dysfunction in human oral mucosa of elderly individuals. To this end, we recruited 32 human participants that included young (age <60) and elderly individuals (age >60), and collected their saliva, peripheral blood mononuclear cells (PBMC) and oral gingival mucosa biopsies. We performed CD4 T cell immunophenotyping in human oral intra-epithelial and lamina propria leukocytes (HOIL) in gingival mucosa from aged and young groups (**Figure S12**). Although the overall CD4$^+$ T cell proportions in HOIL were slightly lower (**Figure S12**, bottom), T_{reg} percentages were significantly higher in elderly individuals compared to the younger group (**Figure 10A**). However, T_{reg} proportions were comparable in PBMC of these groups, again showing a compartmentalized effect on mucosal T_{regs} (**Figure 10A**, bottom). We also observed a blunted expression of CD25 in these T_{reg} cells in aged individuals (**Figure 10A**, X-axis; see legend). Remarkably, examination of Foxp3$^+$ cells showed that aged HOIL had significantly lower proportions of $T_{reg}17$ cells compared to younger counterparts (**Figure 10B**, left). Aged Foxp3$^+$ cells also displayed dampened mTOR phosphorylation in ROR-γt$^+$ fraction (**Figure 10B, middle**), but higher proportions of T_{regDys} cells compared to young Foxp3$^+$ cells (**Figure 10B**, right). Finally, gating on non-Foxp3 CD4$^+$ effector cells, we found that CD4$^+$ T cells from the elderly group showed a significantly higher frequency of IFN-γ expressing effector cells than younger individuals in HOIL (**Figure 10C**). This result suggested that oral mucosal CD4$^+$ T cells in elderly individuals may have heightened activated state, despite an increase in T_{regs}. Again, these alterations showed significant differences in HOIL, but not in PBMC (**Figure 10C**, right). These data suggested a dysregulated MyD88 signaling in T_{regs} due to IL-1β/IL-6 alterations in oral mucosa of aging humans, consistent to what we observed in aged mice. Validating this tenet, IL-1β levels were significantly diminished in saliva from aged individuals, although there were no differences observed in serum (**Figure 10D**). However, IL-6 was found to be elevated in aged individuals in saliva but not in serum (**Figure 10E**), implicating its role in expansion of oral mucosal T_{regDys} cells in aged group (**Figure 10C**). These data concur to the results from mouse experiments, which showed that dysregulation in IL-1β/IL-6 balance may contribute to oral T_{reg} dysfunction in aging mucosa.

DISCUSSION

Our results reveal a previously unknown role of the IL-1β/MyD88/mTOR axis in modulating mucosal T_{regs} during an infection. Although IL-1β is a conventional pro-inflammatory

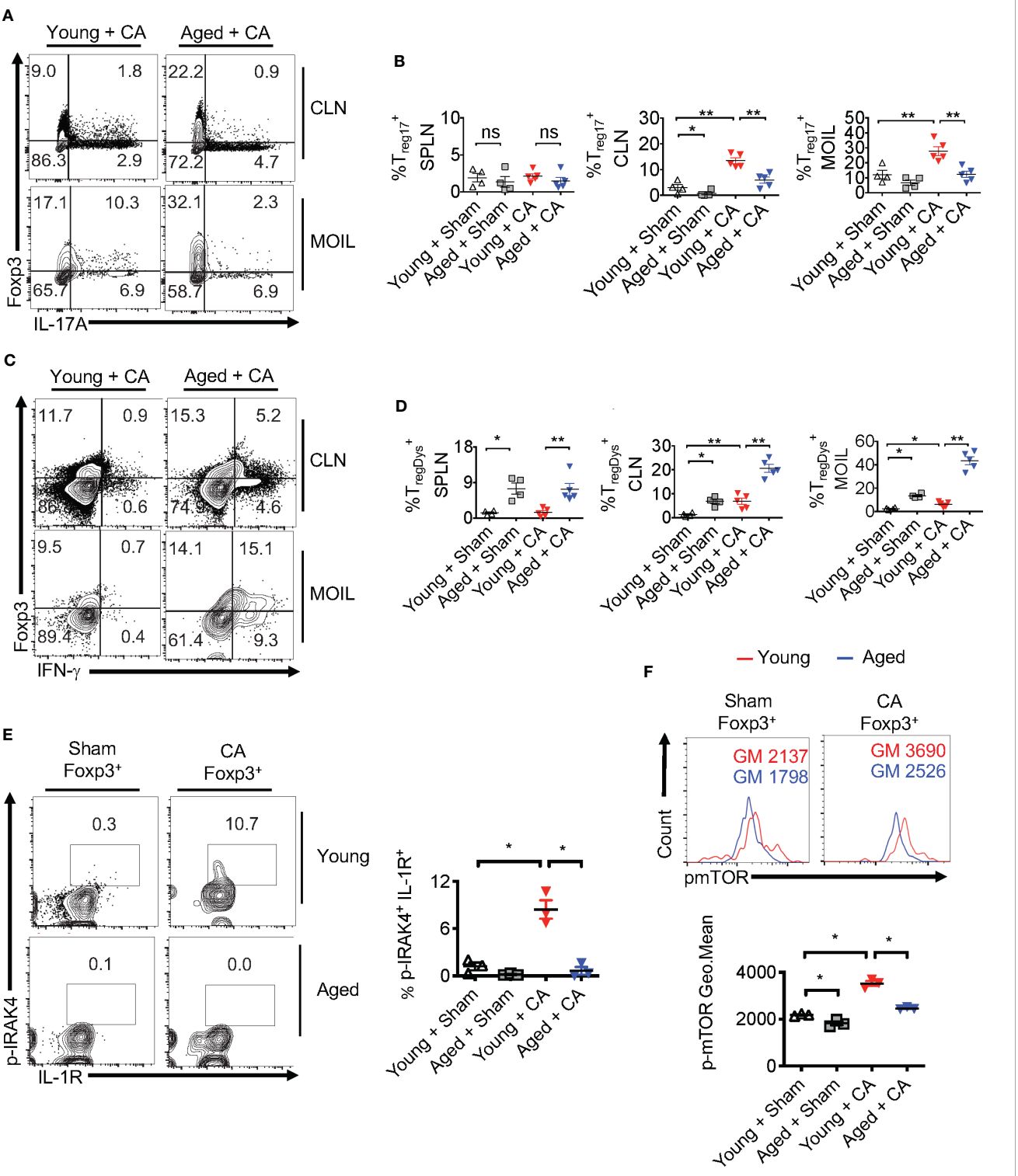

FIGURE 9 | $T_{reg}17$ and T_{regDys} alterations are accompanied by loss of IRAK-4 and mTOR activation in T_{regs} in infected aged mice. Young and Aged mice were infected with CA or Sham as in **Figure 8**. Flow cytometric analyses of $CD3^+CD4^+$ gated cells for IL-17A and Foxp3 **(A, B)**, IFN-γ and Foxp3 **(C, D)**, on day 5 after infection. Statistical analyses of $Foxp3^+$ $IL\text{-}17A^+$ (% $T_{reg}17$) **(B)**, and $Foxp3^+$ IFN-γ^+ (% T_{regDys}) **(D)**, using data points from **(A, B)**, respectively. **(E, F)** Mice were infected for 2 days. Flow cytometry analyses of $CD3^+CD4^+$ $Foxp3^+$ gated cells for p-IRAK and IL-1R (**E**, left and right) and p-mTOR (**F**, top and bottom) in $Foxp3^+T_{regs}$. Geometric mean fluorescence intensities (GM) are indicated in the histogram plots. Mean values ± SEM are plotted in statistical analyses. These data show one of four independent experiments showing similar results. (*P < 0.05; Mann Whitney test). **P < 0.005. NS, non-significant.

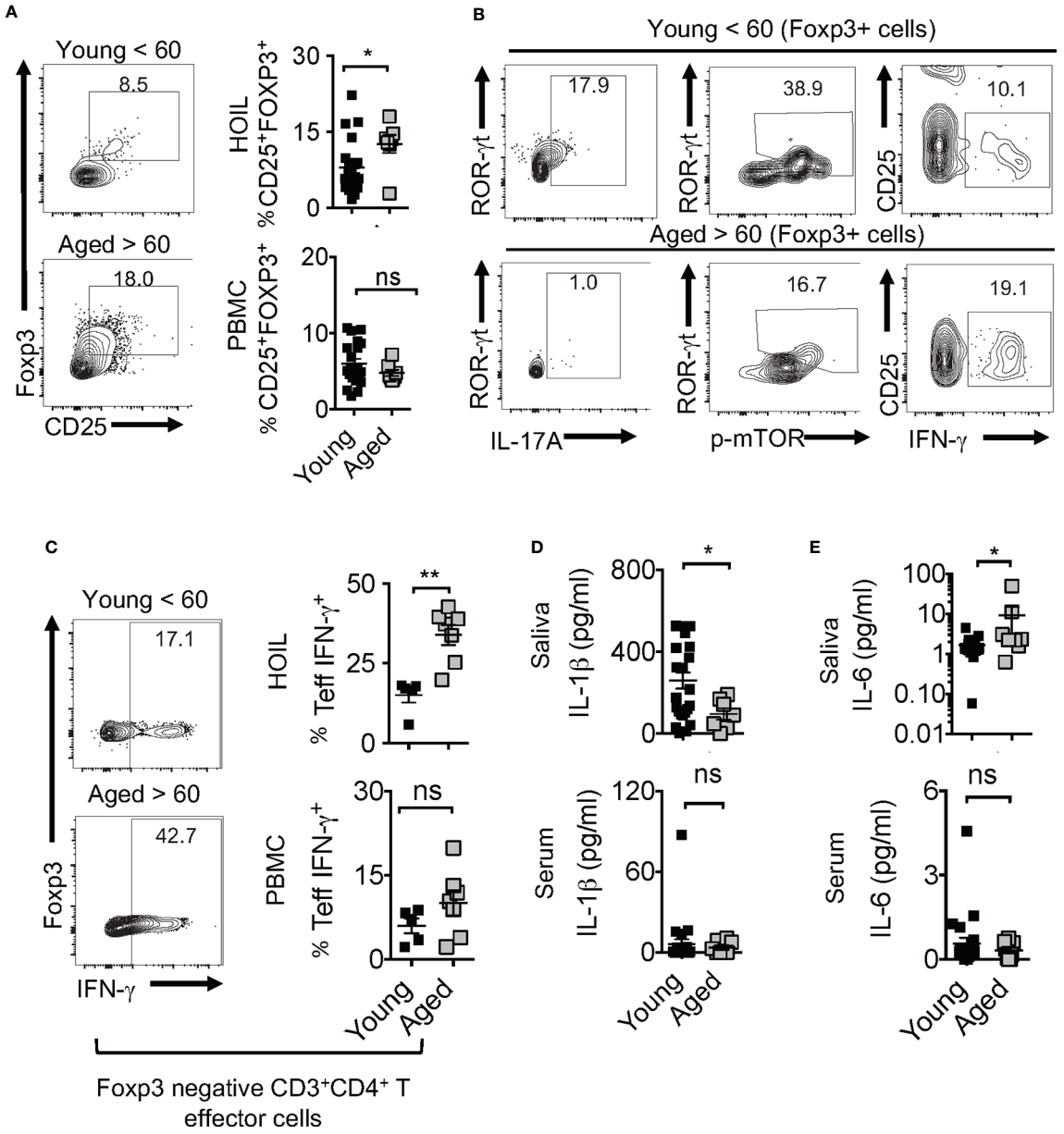

FIGURE 10 | Aged individuals have decreased $T_{reg}17$ cells and increased T_{regDys}, correlating with CD4 T cell hyperactivation in oral mucosa. 32 human participants were recruited to the study (Male n=13, Female n=19; Aged < 60 n = 25; Aged > 60 n=7). Gingival biopsies and peripheral blood mononuclear cells (PBMC) were collected. Human oral intra-epithelial and lamina propria leukocytes (HOIL) and PBMC were obtained by processing gingival biopsy tissues and blood, respectively. Flow cytometric plots showing HOIL Foxp3 and CD25 expression; Geometric Mean of CD25; Young=1387 Aged= 744 **(A)** and statistical analysis of T_{reg} proportions in $CD3^+CD4^+$ of HOIL (above) and PBMC (below) (n=32). **(B)** Flow cytometric plots showing ROR-γt and IL-17A (left), ROR-γt, p-mTOR(middle), CD25, and IFN-γ (right) in HOIL Foxp3+ cells. **(C)** Flow cytometric analyses of non-T_{reg} $CD4^+$ HOIL (above) and PBMC (below) cells showing IFN-γ expression *ex vivo* (n=12) ($CD3^+CD4^+Foxp3^{neg}$ gated). ELISA quantification of IL-6 **(D)**, and IL-1β **(E)**, in saliva (above) and serum (below) collected from the participants. Mean values ± SEM are plotted. (*P < 0.05; Mann Whitney test). **P < 0.005. NS, non-significant.

cytokine, our study unveils an unexpected and surprising role of this cytokine in inducing functionally robust $Foxp3^+$ cells, namely $T_{reg}17$ cells, during the early phases of infection. While TLR-2-MyD88 promotes $Foxp3^+$ cell expansion, IL-1β produced by APC induces IL-17A expression and enhances the proliferation of these T_{regs}. At the cellular level, we show that IL-1β/MyD88 mediated generation of $T_{reg}17$ cells involves the conversion of both conventional $CD4^+$ cells and $Foxp3^+T_{reg}$ cells into $T_{reg}17$ cells in oral mucosa (**Figure S4**, **Figures 1**, **5**, and **6**). Using $MyD88^{-/-}$ T_{regs} we show that $T_{reg}17$ cells are critical for antifungal immunity and constraining inflammatory reactions to *Candida in vivo*. Loss of oral mucosal T_{regs} only in CLN and MOIL, but not in

spleen of MFYcre mice (**Figure 1B**), signifies the compartmentalized effect of MyD88 signals through *Candida* and possibly also the local oral microbiome in mucosa (**Figure 1**). While our previous report demonstrated the role of short chain fatty acids (SCFA) in T_{reg} functions, (27, 31), our current study highlights the role of MyD88. Infection related immunopathology is unlikely a consequence of hyperactivated CD4 cells present in MFYcre mice before infection (**Figure S2**). It is rather a result of T_{reg} specific defects during infection, because the effector CD4 cells in sham mice had comparable levels of cytokines in both FYcre and MFYcre mice (**Figures 3A, B**). However, after the infection, MFYcre mice exhibit excessive proportions of $Ly6C^{high}$ macrophages and Gr-1+ neutrophils, as well as elevated IFN-γ expression in effector CD4+ cells (**Figures 3F–H**). These suggest infection related immunopathology. While we speculate that excessive $CD4^+$IFN-γ may contribute to the persistence of pro-inflammatory monocytes producing IL-6, this possibility remains to be evaluated in the future.

On the one hand, T_{regs} are known to enhance Th17 mediated host immunity and fungal clearance at early time-points during oral *Candida* infection (28). Consistently, absence of MyD88 in T_{regs} resulted in diminution of T_{regs}, decreased Th17 cells, and increased fungal burden (**Figures 2C**, **3A, B**). Therefore, MyD88 is required in T_{regs} for their basal proliferation and enhancing Th17 functions at early time-points of infection. On the other, T_{regs} also reduce Gr-1+ (Ly6G) neutrophils and tongue inflammation at later time-points of infection (21). Concurrently, $CD4^+$ cells with little or no regulation by T_{regs} contributed to hyperactivated CD4 T cells, inflammatory monocytes, neutrophils, and tongue immunopathology in *Candida* infected MFYcre mice (**Figures 2D**, **3C**, **4**). Increased infiltration and persistence of these cells at late-time points led us to infer that these mice suffer immunopathology. While a non-resolving infection could also have resulted in increased infiltration of these cells in MFYcre mice, aging mice did not have increased fungal burden but still displayed tongue pathology. Although IL-1β/MyD88 deficiency in T_{regs} is a common link in poor infection outcomes in our MFYcre and aging infection models, there are distinct differences between them. Increased fungal burden and immunopathology are driven predominantly by reduced T_{reg}/T_{reg}17 numbers and functions in MFYcre mice. Moreover, loss of T_{regs} paralleled with the loss of Th17 cells in these mice. Therefore, immunopathology was driven by reduced antifungal immunity and also impaired ability of T_{regs} to control inflammation. However, aged mice showed increased tongue immunopathology even with an intact ability to clear the fungus. These data are congruent with our results that aged mice have intact $Foxp3^+$ cells and intact Th17 immunity. While mechanisms are unclear, it is tempting to speculate that basal hyperinflammatory state in aging mice might additionally contribute to some of these differences. Higher expression of IFN-γ observed in SPLN, even in the absence of infection, indicates such a hyperinflammatory state in aged mice (**Figure 9D**). Taken together, these results led us to infer that reduced T_{reg}17 cells and increased dysfunctional T_{regs}, but not increased fungal burden alone, contributed to immunopathology outcome during *Candida* infection.

In line with previous studies, the mechanism of T_{reg} functions may involve their ability to consume IL-2 leading to the regulation of IL-2 receptor (CD25) (**Figures 4C, D**) and IFN-γ in $CD4^+$ cells [**Figures 3C**, **9A** (21, 26, 28, 57–59)]. Our current data support the possibility that in the context of early IL-1β expression by APC, T_{reg}/T_{reg}17 mediated IL-2 regulation enhance Th17 cells at early phases. At later phases of infection, T_{reg}17 mediated IL-2 consumption and increased TGF-β1 expression in T_{regs} may downmodulate inflammation (21, 28). However, excessive IL-6 may dysregulate this mechanism of IL-2 consumption mediated immunoregulation by T_{regs}. Supporting this tenet, reduced CD25 expression in aged $Foxp3^+$ cells (**Figure 8D**, X-axis, **Figure 10A**, X-axis) and their likely inability to regulate IL-2 is likely associated with their dysfunction and immunopathology during infection in aged mice (26, 57–59). These data are also in accord with previous reports showing an association of $CD25^{low}$ $Foxp3^+$ cells with tissue autoimmunity (60). The contribution of IL-17A in the immunomodulatory role of T_{reg}17 cells is currently unclear. We cannot rule out the function of this cytokine working in concert with immunomodulatory DC or macrophages unique to the mucosal environment during *Candida* infection (61, 62). While these T_{reg}17 cells appear to be consistent with IL-1R1$^+$$CD25^+$ tissue T_{reg} phenotype (63), other molecular features that are associated with their regulatory function *in vivo* remain to be investigated. Absence of differences in IL-10 expression among $Foxp3^+$ cells between FYcre and MFYcre groups (**Figure S6A**) led us to infer that IL-10 might not be involved in T_{reg}17 mediated immunomodulation in our system.

In light of the current findings we postulate that *Candida* induces mature IL-1β expression in APC which in the context of synergistic IL-1R/TGF-β1 signaling, promotes T_{reg}17 cells. Expression of TLR-2, IL-1R, as well as IL-17A in tT_{regs} and pT_{regs}, verifies this postulate (2). At the molecular level, IL-1β induces the activation of IRAK-4, Akt, mTOR, and p70-S6K axis in naïve $CD4^+$ T cells and $Foxp3^+T_{regs}$ in an IL-1R dependent manner (**Figure 7**). mTOR activation in T_{regs} is crucial for effector T_{reg} function *in vivo* (30, 64). Concurringly, here we show that MyD88/IL-1β mediated mTOR activation induces T_{reg}17 cells *in vitro* and *in vivo* (**Figures 2**, **7**, **10**). Surprisingly, our current study also revealed that IL-1β restrains IL-6 dependent IFN-γ expression in $Foxp3^+$ cells during *Candida* activation (**Figure 6E**). Blocking IL-6 did not alter T_{reg}17 induction in IL-1R$^{-/-}$ cells, which suggests that IL-6 acts synergistically with IL-1β and cannot independently induce IL-17A in $Foxp3^+$ cells. However, the mechanism by which IL-1β interacts with IL-6 signaling and constrains IFN-γ expression in $Foxp3^+$ cells is currently unclear and remains to be studied. Also, the identity of oral mucosal monocytes or macrophages that express active IL-1β/IL-6 during an infection is unknown and warrants future studies (49).

Previous reports show that IFN-γ secretion by $Foxp3^+$ cells is associated with T_{reg} dysfunction (38, 65). Consistently, our study shows that in the context of excessive IL-6, IL-1β/MyD88 deficiency in $Foxp3^+$ cells contributes to their IFN-γ secretion. Such IFN-γ expressing T_{regDys} cells fail to control oral mucosal

inflammation and host defense. While IL-6 is required for Th17 development and resistance to *Candida* infection (66), our data show that elevated levels of IL-6 are strongly associated with Foxp3$^+$ T_{regDys} phenotype and immunopathology in infected aged mice (**Figure 9**). These data are also concurrent with elevated IL-6 observed in patients with chronic mucocutaneous candidiasis (67). Our data also imply that elevated IL-6 and dysbiosis, which might include *Candida* dysbiosis, may contribute to T_{reg} dysfunction and CD4 hyperactivation in aging human oral mucosa (**Figure 10**) (68). Thus, our study supports that IL-1β/IL-6 imbalance is central to Foxp3$^+$ cell plasticity and inflammation control during *Candida* infection (**Figures 8E–H**, **9A**). Although we did not evaluate the effect of microbial dysbiosis in T_{reg} dysfunction in elderly participants, we observed elevated IL-6 levels and CD4$^+$ T cell dysfunction in aging human individuals (**Figure 10**). These data may partially explain the higher prevalence and mortality of *Candida* infections in elderly patients and warrant future studies examining $T_{reg}17$ cells during *Candida* infection. Taken together, our study reveals a central role of IL-1β-mTOR-$T_{reg}17$ axis in controlling oral inflammation and provides an insight into how dysregulation of this mechanism could contribute to overt inflammation during mucosal infections in elderly individuals. It also suggests that manipulating this signaling represents a potential strategy to target T_{reg} functions in mucosa.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB, University Hospitals, CWRU. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by IACUC.

AUTHOR CONTRIBUTIONS

PP designed the study, performed experiments, analyzed data, supervised the project, and wrote the manuscript. FF and AP provided gingival biopsies from human participants. NB and ES performed the experiments, genotyping, and analyzed ELISA and qPCR data, and contributed to discussions. PM performed validation qPCRs and analyzed the data. ES scored the infected mice in a blinded fashion and isolated mouse tissues. *Foxp3 YFP^{cre}* and *Myd88$^{flox/flox}$* mice were bred in NIAID, NIH mouse facility before transferring to the mouse facility at CWRU. SJ performed microscopy of the histology slides and analyzed the data. AW read the manuscript and contributed to discussions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Antigenic Challenge Influences Epigenetic Changes in Antigen-Specific T Regulatory Cells

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Background: Human regulatory T cells (Tregs) are the fundamental component of the immune system imposing immune tolerance *via* control of effector T cells (Teffs). Ongoing attempts to improve Tregs function have led to the creation of a protocol that produces antigen-specific Tregs, when polyclonal Tregs are stimulated with monocytes loaded with antigens specific for type 1 diabetes. Nevertheless, the efficiency of the suppression exerted by the produced Tregs depended on the antigen with the best results when insulin β chain peptide 9-23 was used. Here, we examined epigenetic modifications, which could influence these functional differences.

Methods: The analysis was pefromed in the sorted specific (SPEC, proliferating) and unspecific (UNSPEC, non-proliferating) subsets of Tregs and Teffs generated by the stimulation with monocytes loaded with either whole insulin (INS) or insulin β chain peptide 9-23 (B:9-23) or polyclonal cells stimulated with anti-CD3/anti-CD28 beads (POLY). A relative expression of crucial Tregs genes was determined by qRT-PCR. The Treg-specific demethylated region (TSDR) in FoxP3 gene methylation levels were assessed by Quantitative Methylation Specific PCR (qMSP). ELISA was used to measure genomic DNA methylation and histone H3 post-translational modifications (PTMs).

Results: Tregs $SPEC_{B:9-23}$ was the only subset expressing all assessed genes necessary for regulatory function with the highest level of expression among all analyzed conditions. The methylation of global DNA as well as TSDR were significantly lower in Tregs $SPEC_{B:9-23}$ than in Tregs $SPEC_{INS}$. When compared to Teffs, Tregs were characterized by a relatively lower level of PTMs but it varied in respective Tregs/Teffs pairs. Importantly, whenever the difference in PTM within Tregs/Teffs pair was significant, it was always low in one subset from the pair and high in the other. It was always low in Tregs $SPEC_{INS}$ and high in Teffs $SPEC_{INS}$, while it was high in Tregs $UNSPEC_{INS}$ and low in Teffs $UNSPEC_{INS}$. There were no differences in Tregs/Teffs $SPEC_{B:9-23}$ pair and the level of modifications was low in Tregs $UNSPEC_{B:9-23}$ and high in Teffs $UNSPEC_{B:9-23}$. The regions of PTMs in which differences were significant overlapped only partially between particular Tregs/Teffs pairs.

Conclusions: Whole insulin and insulin β chain peptide 9-23 affected epigenetic changes in $CD4^+$ T cells differently, when presented by monocytes. The peptide preferably favored specific Tregs, while whole insulin activated both Tregs and Teffs.

Keywords: TSDR, antigen-specific, DNA methylation, histone H3, gene expression, epigenetics

INTRODUCTION

T regulatory cells (Tregs) constitute a subset of $CD4^+$ T lymphocytes which is pivotal in immune tolerance due to their ability to suppress effector cells. There are two main subpopulations of Tregs: natural (nTregs or tTregs) which develop in the thymus during thymopoesis and peripheral (pTregs) which differentiate from naïve $CD4^+$T cells in the periphery during TCR stimulation in the presence of cytokines (e.g. IL-2, TGF-β). Natural T regulatory cells ($CD4^+CD25^{high}CD127^-FoxP3^+$; Tregs) are mainly predisposed to exert suppressive functions over effectors, which is highlighted by stable genomic architecture in this subset of Tregs. Transcriptional factor FoxP3 (forkhead box P3) is a master regulator of Tregs. Its expression in Tregs is kept stable *via* Treg-specific demethylated region (TSDR) in the promoter of *FoxP3* gene. The sustained expression of FoxP3, possible due to demethylated TSDR, allows the expression of a wide range of other genes encoding such as: *Eos, GITR, CTLA4*, and simultaneously suppresses activation of: IL-2, IL-4 and INF-γ (1–4). Other important function-associated genes in Tregs are: *IL2RA* (CD25), *CTLA4* (CD152), *TNFRSF18* (GITR), *IKZF2* (Helios), *IKZF4* (Eos) and *Tet2* (5–8).

Tregs ability to prevent excessive immune response has been tested in many clinical trials. In human autoimmune diseases or transplantation, a broken tolerance can be restrained by administration of Tregs. In our hands, the therapy with expanded Tregs was successfully administered in type 1 diabetes or graft versus host disease (GvHD) after bone marrow transplantation (9–11). Until now, mainly polyclonal Tregs have been used in clinical therapies due to the problem in technical expansion of antigen-specific cells (12, 13). Only recently, we have developed a technique, which allows for efficient production of bulk quantities of antigen-specific Tregs, which seems to be a promising tool for autoimmune therapies (14). Our method is based on antigen-loaded monocytes which preferentially activate Tregs specific to presented antigen. Because our work is mainly focused on type 1 diabetes, we used either whole insulin or insulin β chain peptide 9-23 as antigens. Surprisingly, we have found that Tregs generated with β chain peptide 9-23 were significantly more suppressive than those generated with the whole insulin.

Looking for the reasons of such a difference, we examined epigenetic features, presented at **Figure 1**, of both: Tregs ($CD4^+CD25^{high/+}CD127^-$) and T effector cells ($CD4^+CD25^{low/-}CD127^+$; Teffs) generated with monocytes loaded with either whole insulin or insulin β chain peptide 9-23 sorted as antigen-specific (index SPEC) cells. We have also looked at Tregs and

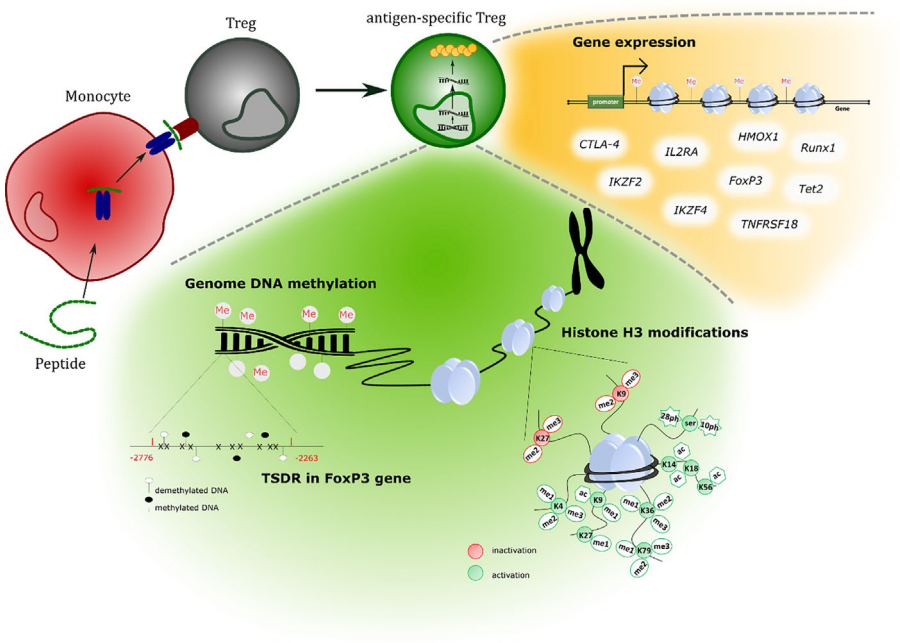

FIGURE 1 | An overview of tests performed to study epigenetic features of generated cells.

Teffs unspecific to the antigens (index UNSPEC) as well as those expanded with anti-CD3/anti-CD28 beads used currently as the polyclonal (index POLY, 1:1 ratio bead:cell) in the treatment of type 1 diabetes. Taking into account already known TSDR-mediated regulation of *FoxP3* gene, we assumed that other epigenetic changes could be also very important in the activity of the manufactured cells and therefore we investigated global genomic DNA methylation, methylation in specific TSDR region of *FoxP3* gene and histone H3 post-translational modifications (PTMs). In addition, we assessed in all subsets the expression of genes crucial in the activity of Tregs, such as: *FoxP3, CTLA-4, IKZF2, IKZF4, IL2RA, TNFRSF18, Tet2, Runx1* and *HMOX1*. Indeed, we found significant differences between the subsets, which could impact the activity of the cells.

MATERIALS AND METHODS

Research Material

Buffy coats, with unknown HLA, were obtained from the Regional Centre for Blood Donation and Treatment in Gdańsk from volunteers donating blood. All tests were conducted on male volunteers aged 18-65.

Cells Preparation

Detailed procedure for cells preparation was described by Iwaszkiewicz-Grzes D. et al. previously (14). General procedure is presented at the workflow in **Figures 2** and **S1**.

Tregs and Teffs were freshly isolated from buffy coats obtained from anonymous healthy volunteer blood donors according to previously described protocol (15). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by gradient centrifugation using Ficoll-Paque Plus. Collected PBMCs were counted and separated into two tubes, first tube for isolation of $CD4^+$ cells using negative immunomagnetic selection method (StemCell EasySep™ Human CD4 Negative Selection Kit, StemCell Technologies, Canada) and second tube for isolation of CD14+ cells using positive immunomagnetic selection method (StemCell EasySep™ Human CD14 Positive Selection Kit, StemCell Technologies, Canada).

$CD4^+$ T cells were transferred into cell culture flask in X-VIVO culture medium with addition of penicillin/streptomycin and remained to the next day under standard conditions (37°C, 5% CO_2, 95% O_2). At the same time, $CD14^+$ monocytes (Mo) were isolated according to the manufacturer's instructions. Next, $CD14^+$ cells were suspended in X-VIVO culture medium (Lonza), spread out into plates and stimulated for 24h with tested antigens: whole insulin (index INS, 350µg/well/ml (16), Actrapid® Penfill®, Novo Nordisk A/S) and insulin β chain peptide (index B:9-23, 25µg/well/ml; Lipopharm; Gdansk, Poland) under standard conditions (37°C, 5% CO_2, 95% O_2).

After 24h $CD4^+$ T cells were stained with monoclonal antibodies (mAbs): CD3, CD4, CD25 and CD127, and sorted with FACS AriaII sorter (BD Biosciences, USA) into Tregs ($CD3^+CD4^+CD25^{high/+}CD127^{-/low}lin^-doublet^-$) and Teffs ($CD3^+CD4^+CD25^{low/-}CD127^+lin^-doublet^-$).

Next, Tregs and Teffs were stained with violet (Cell Trace Violet Cell Proliferation Kit, Life Technologies, 1µM; 15min, 37°C) (17). At the same time, previously prepared monocytes were collected and irradiated.

We prepared the following conditions: Tregs/Mo stimulated with INS or B:9-23, Tregs/beads (ExpAct Treg Beads conjugated to CD28, Anti-Biotin, and CD3-Biotin monoclonal antibodies; MACS®GMP; in 1:1 ratio (bead:cell)), Teffs/Mo stimulated with INS or B:9-23 and Teffs/beads in X-VIVO culture medium with addition of IL2 (100 IU/ml), heat-inactivated human serum (10%) and penicillin/streptomycin. Cultures containing monocytes were additionally stimulated with anti-CD28 and anti-CD154 antibodies at a final concentration of 5µg/ml/well each (BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD154/CD28). After 6 days of expansion cells were collected and sorted based on violet fluorescence (**Figure S1**) (17). Cells responding to the antigen presented by the monocytes were identified as antigen-specific (index SPEC), non-proliferating cells were identified as unspecific (index UNSPEC). During whole procedure cells were stimulated only once with monocytes. Cells stimulated with beads were treated as polyclonal (specific against many antigens, index POLY).

Obtained cells were expanded with beads (no longer than 5 days) in order to obtain enough cells for all tests, at least 1 million cells per condition (Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC) for cells stimulated with whole insulin and insulin β chain peptide 9-23 and stored in -70°C maximum 1 month.

RNA Extraction and RT-qPCR

Total RNA was isolated using AllPrep® DNA/RNA Mini Kit (Qiagen, USA) following the manufacturer's instruction. Assessment of RNA concentration and purity was measured *via* spectrophotometer (Epoch, BioTek). Obtained RNA was stored in -70°C until use.

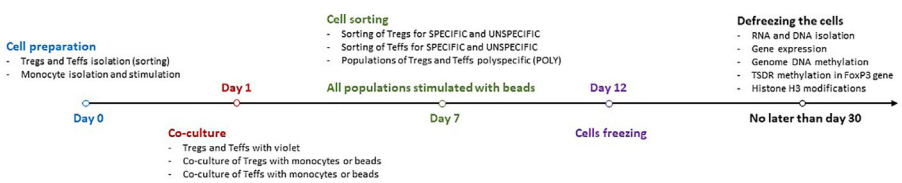

FIGURE 2 | The scheme of obtaining cells and their further use.

500ng total RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) under standard conditions: step 1 - 25°C/10 min; step 2 - 37°C/120 min; step 3 - 85°C/5 min; step 4 - 4°C/∞ min. The expression of target genes, characteristic for Tregs: *FoxP3, IKZF2, IKZF4, IL2RA, TNFRSF18, Tet2, Runx1* and *HMOX1* was detected using FastStart Essential DNA Probes Master (Roche, Switzerland) on LightCycler®96 (Roche, Switzerland) in accordance to the manufacturer's protocols in prepared cell populations (Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC). The primer sequences (Sigma-Aldrich) and used probes (Universal ProbeLibrary Set, Human with Probes; Roche, Switzerland) were designed using Assay Design Center Software (Roche) and are listed in **Table 1**. GAPDH was used as the housekeeping gene and the normalized expression ratio of the target genes in prepared cell populations was calculated using the $2^{-\Delta\Delta Ct}$ (Livak method) (18). All reactions were carried out in triplicate from three independent experiments.

Genomic DNA Extraction and Global DNA Methylation

DNA was isolated using AllPrep® DNA/RNA Mini Kit (Qiagen, Germany) following the manufacturer's instruction from following cells: Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC for cells stimulated with whole insulin and insulin β chain peptide 9-23. Assessment of DNA concentration and purity was measured *via* spectrophotometer (Epoch, BioTek). Obtained DNA was stored in -20°C until use.

Quantification of genomic DNA methylation was performed using Methylated DNA Quantification Kit (Colorimetric) (Abcam, UK). 100 ng/μl of DNA was used per reaction (well) under manufacturer's instruction. Absorbance was read at 450 nm *via* Epoch (BioTek) spectrophotometer with Gene5 software. Obtained absorbance was used to calculate percentage of genomic DNA methylation in each cell population. The total/global amount of methylated DNA was calculated by generation of a standard curve. Next, the slope (OD/ng) of the standard curve was determined using linear regression and then the analysis of absolute/total quantification of 5-mC in total DNA was determined.

Histone Extraction and Histone H3 Modification

Cells stimulated with beads, whole insulin or insulin β chain peptide 9-23 (Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC) were pelleted and histones isolated with Histone Extraction Kit (Abcam, UK) according to the manufacturer's instructions. Two Assay Control Proteins were prepared with the final concentrations of 5ng/μl and 25ng/μl. 150ng of histone extract per well for each modification were used in triplicate. Assessment of histone concentration and quality were measured *via* spectrophotometer Epoch (BioTek). 21 histone H3 modifications, which include the most important and the most well characterized patterns, were measured using Histone H3 Modification Multiplex Assay Kit (Colorimetric, Abcam) and are listed in **Table 2**. Obtained absorbance was used to calculate % of individual histone H3 modifications.

DNA Bisulfite Conversion and Methylation-Specific PCR

Quantitative methylation-specific polymerase chain reaction with methylated (M) and unmethylated (U) primers was used for detection of methylation of the TSDR region in *FoxP3* gene. Briefly, genomic DNA was extracted from maximum 1 million of cells using AllPrep® DNA/RNA Mini Kit (Qiagen, Germany) and submitted to bisulfite conversion using the EpiTect® Bisulfite Kit (Qiagen, Germany) under manufacturer's instruction. First, DNA bisulfite conversion was performed in which unmethylated cytosine

TABLE 1 | Sequences of primers used for real-time PCR.

Gene Name	RefSeq Accession Number	Primer	Amplicon Size (nt)	%GC	Tm	Primer Sequence (5'-3') Forward	Probe #
GAPDH*	NM_001289745.1	Fw	70	45	60	CCCCGGTTTCTATAAATTGAGC	#75
		Rv		58	59	GGCTGACTGTCGAACAGGA	
FoxP3	NM_014009.3	Fw	102	55	59	ACACTGCCCCTAGTCATGGT	#25
		Rv		50	60	GAGCTGGTGCATGAAATGTG	
CTLA-4	NM_005214.4	Fw	65	56	60	TGGGTCCCAGGGAAGTTT	#25
		Rv		50	60	TGACCTTGTGTTCTACCTGGTG	
IKZF2	NM_016260.2	Fw	64	45	59	CATCACATTGCTTTGCCCTA	#61
		Rv		48	59	TCATCACTGTCAGAGAGAGGCTA	
IKZF4	NM_022465.3	Fw	68	45	60	TCAGGCATTTGTTGTGCAGT	#3
		Rv		53	59	AGGGAAAGGCAGATGCTGT	
IL2RA	NM_000417.2	Fw	73	55	59	CCAACTTCCCAGTTCAGGAG	#45
		Rv		44	59	GGGTAGAGTGTGTGTGTTGTGTATT	
TNFRSF18	NM_004195.2	Fw	92	61	59	ACCTGGGTCGGGATTCTC	#10
		Rv		61	59	CACAGCCAGTTGGACACG	
Tet2	NM_001127208.2	Fw	93	36	59	AAAGATGAAGGTCCTTTTTATACCC	#68
		Rv		48	59	ACCCTTCTGTCCAAACCTTTC	
Runx1	NM_001754.4	Fw	61	41	60	CCAAAGAGTGTGGAATTTTGGT	#55
		Rv		50	59	AAACAGGGCGAGTTGCAT	
HMOX1	NM_002133.2	Fw	61	55	59	CCCTTCAGCATCCTCAGTTC	#84
		Rv		58	59	GACAGCTGCCACATTAGGG	

**GAPDH was used as an endogenous control.*

TABLE 2 | Histone H3 modifications.

Methylation	Acetylation	Phosphorylation
H3K4me1↑	H3K9ac↑	H3ser10ph↑
H3K4me2↑	H3K14ac↑	H3ser28ph↑
H2K4me3↑	H3K18ac↑	
H3K9me1↑	H3K56ac↑	
H3K9me2↓		
H3K9me3↓		
H3K27me1↑		
H3K27me2↓		
H3K27me3↓		
H3K36me1↑		
H3K36me2↑		
H3K36me3↑		
H3K79me1↑		
H3K79me2↑		
H3K79me3↑		

↑ *activating modification.*
↓ *inactivating modification.*

residues are deaminated to uracil and methylated cytosine (5-mC) residues remain intact. 500 ng of isolated DNA per reaction were used. Bisulfite reaction was performed in the thermocycler with the following parameters: 95°C for 5 min, 60°C for 25 min, 95°C for 5 min, 60°C for 85 min, 95°C for 5 min and 60°C for 175 min. After conversion, DNA was subjected to quantitative methylation-specific PCR procedure using TB Green Premix Ex Taq II (Takara, Japan) on LightCycler®96 (Roche, Switzerland) in accordance with the manufacturer's protocols. EpiTect® PCR Control DNA Set (Qiagen, Germany) was used as positive and negative controls. Unconverted DNA was considered as a negative control. Methylated and unmethylated primers for *FoxP3* gene intron 1 were designed using MethPrimer 2.0 Software by Zafari et al. and are listed in **Table 3** (19, 20). Real-time PCR was performed in a final reaction volume of 20 µl using the Roche Life Science LightCycler® 96 including 5 pmol of each forward and reverse methylated/demethylated primer and 50–100 ng of bisulfite-treated genomic DNA. PCR consisted of an initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, followed by 60°C for 30 s, 1 cycle of melting at 95°C for 5 s, followed by 60°C for 1 min, 1 cycle of cooling at 50°C for 30 s. The level of methylation was also verified by electrophoresis on a 2% agarose gel using 100 bp DNA Ladder (Invitrogen, USA) as a marker.

Statistics

The Statistica 13.0 software was used to perform all statistical analysis. Significance was calculated using the *t*- test. Significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

RESULTS

Influence of Antigen Stimulation on Gene Expression

In the current study we selected several genes known to be involved in the function of Tregs and investigated their expression in particular subsets of cells. We compared polyclonal Tregs, which are a cellular medicinal product used in the treatment of type 1 diabetes, with Tregs specific and unspecific against whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). Polyclonal Tregs were treated as a reference for other cells. To obtain fully valuable results, we also performed the same tests for effector T cells: polyclonal, specific and unspecific against presented antigen. The obtained results are presented in **Figure 3**, and the p values of statistical significance in **Table S1**.

Figure 3A shows gene expression in all populations in a form of a heatmap. With the exception of the IKZF4 gene, Tregs showed higher expression of the studied genes than Teffs. Teffs $SPEC_{INS}$ were the only ones among the effectors which showed low but noticeable expression of IKZF4, TNFRSF18 and HMOX1 genes. Among Tregs subsets (**Figure 3B**), Tregs $SPEC_{B:9\text{-}23}$ and Tregs POLY were of special interest as they expressed all analyzed genes. Tregs $SPEC_{B:9\text{-}23}$ showed the highest levels of expression of RUNX1 and IKZF4 genes but the expression of other genes was moderate. On the other hand, Tregs $SPEC_{INS}$ were the cells with the lowest expression of IKZF2, FoxP3, IKZF4, Tet2 and RUNX1 genes. Tregs $UNSPEC_{INS}$ also showed low expression of the majority of genes, especially IL2RA, HMOX1 and FoxP3, when compared to Tregs $SPEC_{B:9\text{-}23}$.

We could observe statistically significant differences between particular subsets in the expression of five genes: FoxP3, IKZF2, IKZF4, IL2RA and TNFRSF18 which are presented separately in **Figure 3C**. Values of statistical significance for gene expression are presented in **Table S1**.

The Influence of Antigen Stimulation on Genomic DNA Methylation

Based on a colorimetric assay for quantification of global DNA methylation by measuring levels of 5-methylocytosine (5-mC) we observed statistically significant differences between cells (**Figure 4**). Tregs $SPEC_{INS}$ and Tregs $UNSPEC_{B:9\text{-}23}$ had the highest level of methylation. Tregs $SPEC_{B:9\text{-}23}$ were much less affected. Their methylation differed significantly from Tregs $UNSPEC_{B:9\text{-}23}$ (t-test; $p < 0.0001$), Tregs $SPEC_{INS}$ (t-test; $p < 0.0001$) and Teffs $SPEC_{B:9\text{-}23}$ (t-test; $p < 0.0001$). Tregs

TABLE 3 | Primers for TSDR analysis (19).

Primer set	Primer	Primer Sequence (5'-3') Forward	PCR product size	Tm
demethylated	Fw	GGATAGGGTAGTTAGTTTTTGGAATG	117	62.6
	Rv	CCACCATTAACATCATAACAACCA		64.1
methylated	Fw	GATAGGGTAGTTAGTTTTCGGAAC	116	59.9
	Rv	CCGCCATTAACGTCATAACG		64.9

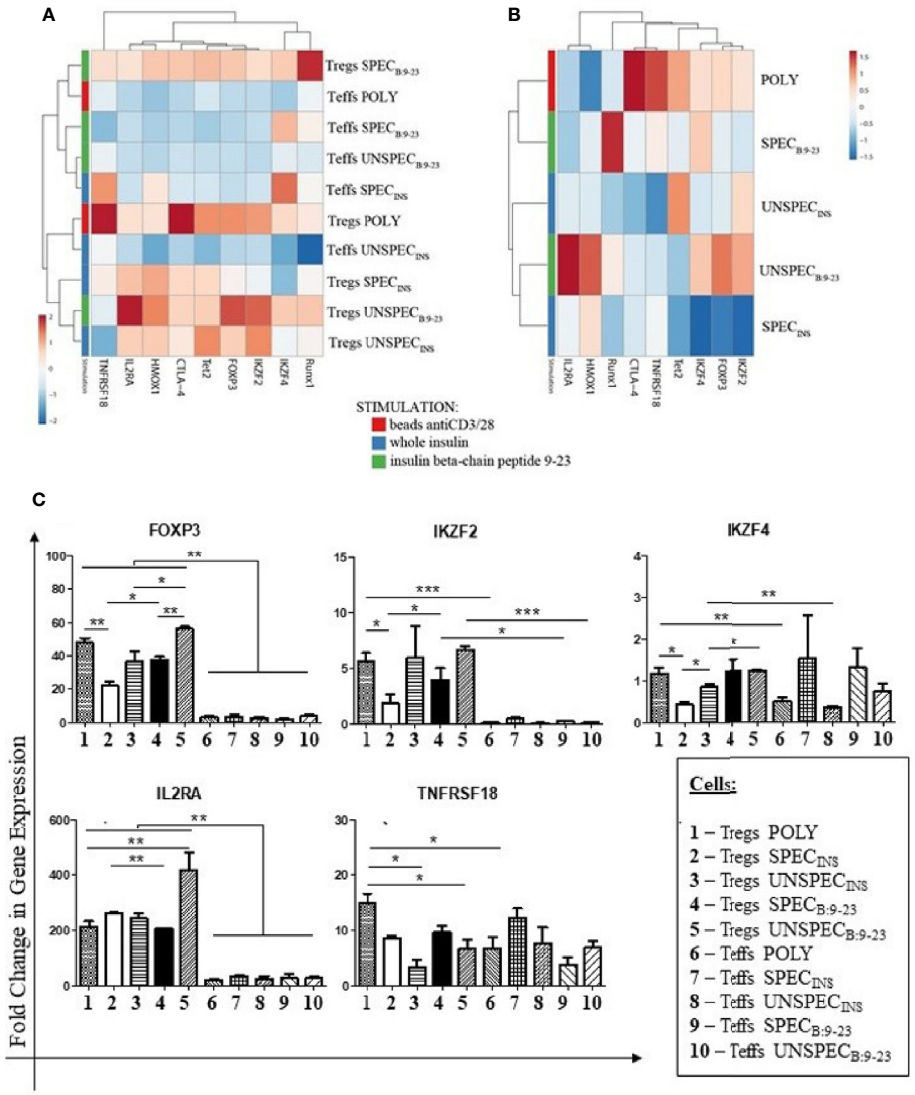

FIGURE 3 | Fold Change in Gene Expression in Tregs and Teffs. **(A):** Clustering and heat maps analysis of gene expression in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. **(B)** Clustering and heat maps analysis of gene expression only in Tregs. **(C)** Graphs presenting changes in cells populations. Only the expressions in which statistical significance occurred are presented. The cells were stimulated with beads anti-CD3/anti-CD28 (index POLY) or monocytes loaded with whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). The cells responding to antigen have an index SPEC, cells not recognizing the antigen have an index UNSPEC. All stimulations were performed in triplicates (three tests for whole insulin and three tests for insulin β chain peptide 9-23). The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). Heat maps were prepared using ClustVis tool based on correlation distance and average linkage between clusters (21).

$SPEC_{B:9-23}$ were also significantly different from antigen-specific Teffs stimulated with the same antigen (Teffs $SPEC_{B:9-23}$; t-test; $p<0.0001$). We did not observe significant differences between Tregs POLY and other cells.

The Influence of Antigen Stimulation on TSDR Methylation in FoxP3 Gene

We next used quantitative methylation-specific polymerase chain reaction with methylated and unmethylated primers for detection of methylation in TSDR region of FoxP3 gene. TSDR in all Tregs showed a level of demethylation over 75%, which was significantly more demethylated than TSDR of Teffs (t-test; $p<0.0001$) (**Figure 5A**). These results were also confirmed by agarose gel electrophoresis (**Figure 5B**). Tregs $SPEC_{INS}$ showed the lowest level of demethylation (75%) among Tregs subsets. It was significantly less compared to Tregs $UNSPEC_{INS}$ (t-test; $p=0.0020$) and Tregs $SPEC_{B:9-23}$ (t-test; $p=0.0065$). Tregs POLY, whose demethylation was ≈80%, were significantly less demethylated than each of the three subsets of Tregs: Tregs $SPEC_{B:9-23}$ (t-test; $p=0.0229$), Tregs $UNSPEC_{B:9-23}$ (t-test;

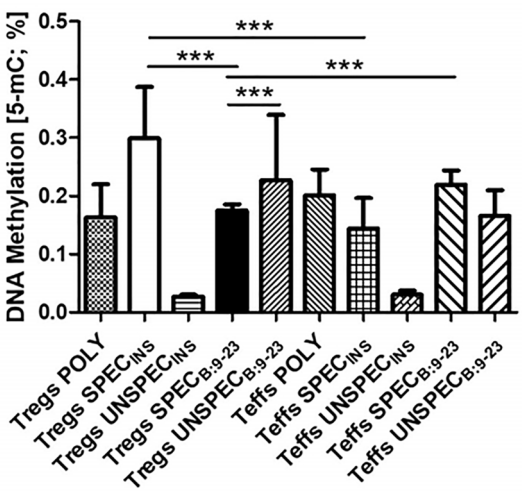

FIGURE 4 | Global DNA methylation in Tregs and Teffs. Comparison of 5-methylocytosine (5-mC) production in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. During the tests cells were stimulated with beads anti-CD3/ant-CD28 (index POLY) or monocytes loaded with whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). Cells responding to antigen have an index SPEC, cells not recognizing the antigen have an index UNSPEC. All analyzes were performed for six tests (three tests for whole insulin and three tests for insulin β chain peptide 9-23) in triplicate. The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with * (p<0.05), ** (p<0.01) or ***(p<0.001).

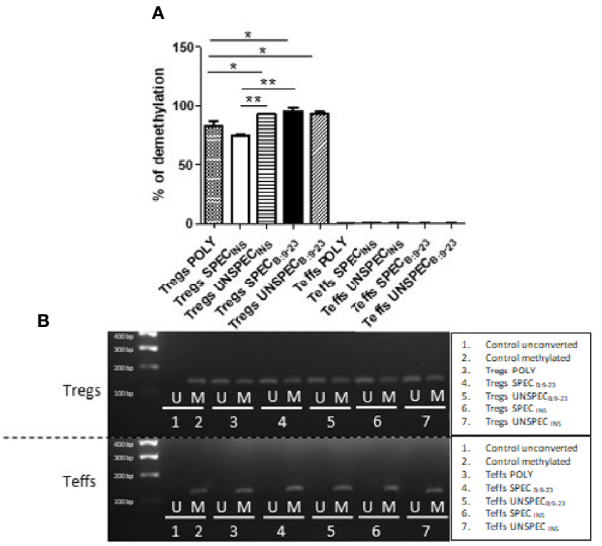

FIGURE 5 | FoxP3 demethylation in TSDR region in Tregs and Teffs. **(A)** Comparison of percentage of demethylation in Treg-specific demethylated region (TSDR) in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. **(B)** Representative results showing FoxP3 demethylation in all cell cultures was detected by agarose gel electrophoresis [reaction with methylated (M) and unmethylated (U) primer]. During the tests cells were stimulated with anti-CD3/anti-CD28 beads (index POLY) or monocytes loaded with whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). Cells responding to the antigen have an index SPEC, cells not recognizing presented antigen have an index UNSPEC. All analyzes were performed for six tests (three tests for whole insulin and three tests for insulin β chain peptide 9-23) in triplicate. The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with *(p<0.05), **(p<0.01) or ***(p<0.001).

p=0.0433) and Tregs UNSPEC$_{INS}$ (t-test; p=0.0451). All these three remaining Treg subsets showed demethylation over 90%.

The Influence of Antigen Stimulation on Histone H3 Modifications

The total concentration of histone H3 protein and individual modifications in ng was calculated and compared at heat-maps (**Figure S2**). Then, based on Total H3, the percentage of different cell modifications was determined (**Figure 6**). We analyzed gene activating modifications (↑) such as methylation: H3K4me(1-3), H3K9me1, H3K27me1, H3K36me(1-3), H3K79me(1-3); acetylation: H3K9ac, H3K14ac, H3K18ac, H3K56ac; phosphorylation: H3ser28P, H3ser10P and gene inactivating modifications (↓) such as methylation: H3K9me2, H3K9me3, H3K27me2, H3K27me3 (**Table 2**). All cultures were performed in triplicates.

The comparison of the precentage of histone H3 modifications in total H3 between Tregs and Teffs (**Figure 6A**) confirmed that the populations of Tregs showed opposite pattern of modifications than Teffs. Teffs, mainly Teffs SPEC$_{B:9-23}$, Teffs SPEC$_{INS}$ and Teffs UNSPEC$_{B:9-23}$, were characterized by a relatively high level of modifications.

While studying the differences between differently stimulated Tregs (**Figure 6B**), Tregs POLY exhibited the highest level of histone modification, when compared to monocyte stimulated cells. When comparing antigen-specific subsets, we could observe that Tregs SPEC$_{INS}$ showed a lower level of modification than Tregs SPEC$_{B:9-23}$, excluding H3K18ac, H3K9me1, H3K9me3 and H3K36me2.

Interestingly, when compared to Teffs, Tregs were characterized by a relatively lower level of PTMs but it varied in respective Tregs/Teffs pairs (**Figure 6C**, **Table 4**). Importantly, whenever the difference in PTMs within Tregs/Teffs pair was significant, it was always low in one subset from the pair and always high in the other. The level of modifications in Tregs SPEC$_{INS}$ was significantly lower than that in Teffs SPEC$_{INS}$ in 7 out of 11 regions, in which any significant differences occurred (H3K4me3, H3K9me2, H3K27me1, H3K79me1, H3K79me3, H3K14ac and, H3ser28P). At the same time, there was no single modification, which level was different between Tregs SPEC$_{B:9-23}$ and Teffs SPEC$_{B:9-23}$ (**Figure 6C**). Tregs UNSPEC$_{INS}$ showed a significantly higher degree of modifications than Teffs UNSPEC$_{INS}$ in 5 out of 11 regions, in which any significant differences occurred (H3K9me2, H3K27me3, H3K79me1, H3K79me3 and, H3K14ac) (**Figure 6C**). The level of modifications in Tregs UNSPEC$_{B:9-23}$ was significantly lower than that in Teffs UNSPEC$_{B:9-23}$ in 6 out of 11 regions, in which any significant differences occurred (H3K27me3, H3K36me1, H3K79me1, H3K9ac, H3K14ac and, H3ser28P). Interstingly, H3K79me1 was the only one modified

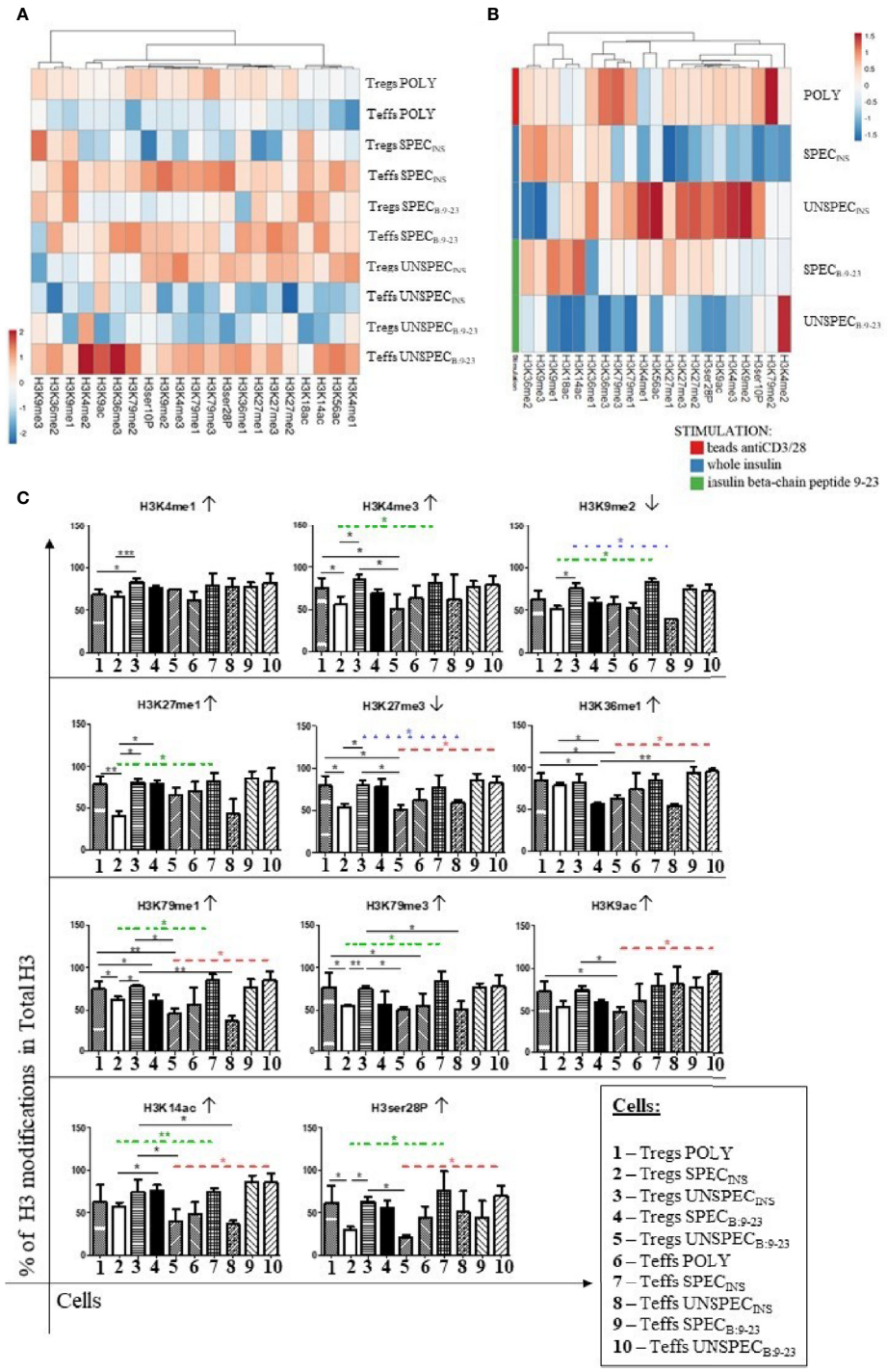

FIGURE 6 | Percentage of histone H3 modification in total H3 in Tregs and Teffs. **(A)** Comparison of percentage of modifications in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. **(B)** Comparison of modifications only in Tregs. Cells were stimulated with anti-CD3/anti-CD28 beads (index POLY) or monocytes loaded with whole insulin (INS) or insulin β chain peptide 9-23 (B:9-23). **(C)** Graphs presenting changes in cells populations. Only modifications in which statistical significance occurs are presented. Cells responding to the antigen have an index SPEC, cells not recognizing the antigen have an index UNSPEC. All analyzes were performed for six tests (three tests for whole insulin and three tests for insulin β chain peptide 9-23) in triplicate. The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). Heat maps were prepared using ClustVis tool based on correlation distance and average linkage between clusters (21). ↑ - activating modification; ↓ - inactivating modification; ···· difference between specific Tregs and Teffs stimulated with insulin; ···· difference between unspecific Tregs and Teffs stimulated with insulin; ···· difference between unspecific Tregs and Teffs stimulated with peptide 9-23.

TABLE 4 | Selectivity index (SI) for histone modifications.

Histone modification	SI [Teffs/Tregs]				
	POLY	$SPEC_{INS}$	$UNSPEC_{INS}$	$SPEC_{B:9-23}$	$UNSPEC_{B:9-23}$
H3K4me1	0,90	1,22	0,93	1,02	1,11
H3K14ac	0,78	**1,31**	0,49	1,13	**2,16**
H3K56ac	0,81	1,22	0,72	1,05	1,56
H3K4me3	0,84	**1,44**	0,73	1,12	1,59
H3K9me2	0,83	**1,64**	**0,51**	1,28	1,28
H3K27me1	0,89	**2,00**	0,53	1,08	1,24
H3K27me3	0,79	1,43	**0,75**	1,09	**1,62**
H3K36me2	0,78	1,07	0,60	1,06	1,37
H3K79me1	0,75	**1,37**	0,47	1,26	**1,86**
H3K79me3	0,70	**1,56**	0,67	1,35	1,56
H3ser28P	0,72	**2,55**	0,80	0,79	**3,30**
H3K4me2	0,92	1,16	0,82	0,98	1,10
H3K9me1	0,79	1,11	0,82	0,93	1,40
H3K9me3	0,95	0,97	1,07	0,83	1,19
H3K27me2	0,80	0,98	0,61	1,14	1,12
H3K36me1	0,88	1,08	0,67	1,69	**1,53**
H3K36me3	0,93	1,13	1,04	1,19	1,30
H3K79me2	0,75	1,18	0,87	1,11	1,27
H3K9ac	0,84	1,48	1,08	1,29	**1,93**
H3K18ac	0,91	1,02	0,91	0,90	1,36
H3ser10P	0,79	1,51	0,88	1,28	1,14

The selectivity index was calculated according to formula $SI = \frac{Teffs}{Tregs}$, where:
Teffs – percentage of histone H3 modification in total H3 in T effector cells.
Tregs – percentage of histone H3 modification in total H3 in T regulatory cells.
green - difference between specific Tregs and Teffs stimulated with insulin;
blue - difference between unspecific Tregs and Teffs stimulated with insulin;
red - difference between unspecific Tregs and Teffs stimulated with peptide 9-23.

region, in which the significant differences were found between all three respective Tregs/Teffs pairs.

Modifications with significant differences are shown in **Figure 6C** and p values in **Table S2**. Modifications without correlations are presented in supplementary materials at **Figure S3**.

DISCUSSION

In this study we aimed to examine the epigenetic background of the difference in the activity between Tregs expanded with monocytes loaded with different peptides, such as whole insulin or insulin β chain peptide 9-23. We have found different pattern of histone PTMs and different level of DNA methylation as well as different expression of genes crucial for Tregs development and suppressive function between the subsets expanded with these different antigens.

Until now, polyclonal T regulatory cells have been used in many clinical trials as a potent medicinal product that downregulates immune response during autoimmune diseases (9). Polyclonal cells, obtained by anti-CD3 and anti-CD28 stimulation, exert positive effect on patients with type 1 diabetes and significantly reduce the inflammatory response (16). One attempt to improve this therapy is to use specific Tregs directed toward disease-causing antigens. Such Tregs should traffic only into the inflamed tissue and suppress autoreactive lymphocytes *in situ* by response against specific antigens. Such an antigen-specific preparation may improve effectiveness of the currently administered treatment with polyclonal Tregs, reduce the required dose and limit adverse effects related to the interaction of Tregs with distant unrelated tissues. In our previous study, we proved that Tregs responding to a particular antigen showed higher potency to suppress Teffs than polyclonal cells. Antigen-specific Tregs retained a higher level of $FoxP3^{high}$ expression and also maintained suppressive phenotype, which makes them more potent to surpass the excessive immune response during autoimmunity (14). Interestingly, Tregs specific to insulin β chain peptide 9-23 were more suppressive than those generated with whole insulin. We found that antigen-specific Teffs could be generated with monocytes loaded with antigens, too. These results are important as they confirmed our *in vivo* data from type 1 diabetes in which we found that the disease-specific antigens can induce both specific Tregs and Teffs and the balance between these two subsets might be associated with the course of the disease (22). Importantly, current report confirms *in vitro* that the whole insulin is a poor stimulator of Tregs and the efficient induction of tolerance should be performed with other peptides, like β chain peptide 9-23.

It is highly interesting which epigenetic changes are exerted by the particular stimuli. It is widely known that the sustained suppressive phenotype of Tregs requires progressive demethylation in Tregs-specific signature genes (23). The majority of the studies focus on *FoxP3* gene and its regulation, because *FoxP3* is a master regulator that provides Tregs function and ensures phenotype maintenance. However, recent data shows that *FoxP3* expression alone is unable to preserve Tregs function without acquisition of Treg-specific epigenome. It is well-established that Tregs deprived of CNS2 or Tregs with high TSDR methylation lack *FoxP3* expression and suppressive function and could even acquire abilities to produce pro-inflammatory cytokines (24, 25). TSDR demethylation within the first intron of *FoxP3* gene locus is specific for Tregs, while in Teffs this region is highly methylated. The analysis of methylation status is reliable and correlates with the generation of stable Tregs (26, 27). The state of demethylation is needed for binding with other transcription factors such as: CREB, NFAT, RUNX to enable *FoxP3* expression (28). The research conducted by Miyao et al. (29) has revealed that TSDR demethylation acts as epigenetic memory that provides linage stability, even in the environment that contributes to *FoxP3* downregulation and thus indicate that stable CNS2 ensures Tregs persistence. In a similar study on effectiveness of antigen-specific Tregs obtained after stimulation with APCs, scientists confirmed that antigen-specific Tregs possessed a comparable average demethylation level (range 70,1-95.2%) to polyclonal cells, while specific and polyclonal Teffs had less than 0,2% of demethylation (30). For these reasons, the measurement of TSDR demethylation was applied as a useful quality control tool in the manufacturing of expanded polyclonal Tregs product (15, 31). In our research, all Tregs showed demethylation over 75% and Teffs were almost 100% methylated, which indicates that during cell culture all Tregs

remained stable. Worth emphasizing are Tregs $SPEC_{B:9-23}$, whose demethylation was significantly higher than Tregs $SPEC_{INS}$ and POLY, which confirms their usefulness as a drug candidate superb to polyclonal Tregs.

The process of genomic DNA methylation is rapid and flexible during T cell activation and differentiation (32). 5'-methylcytosine (5-mC) depletion is a hallmark of active transcription and it is involved in determination of lymphocyte function (33). In our study we examined total percentage of DNA methylation in T lymphocytes and we saw various pattern of methylation in particular subsets. Nevertheless, Tregs $SPEC_{B:9-23}$ were significantly less methylated than Tregs $SPEC_{INS}$. We performed correlation analysis between global DNA methylation and TSDR methylation, and we did not observe any statistical significance. However, we can notice a trend toward increased DNA methylation and diminished TSDR demethylation in Tregs $SPEC_{INS}$, and low global methylation and substantial TSDR demethylation in Tregs $UNSPEC_{INS}$. Moreover, higher % of total DNA methylation does not affect TSDR demethylation in Tregs $UNSPEC_{9-23}$.

Histone PTMs play a major role in chromatin remodeling, due to changes in electrostatic charge of histone protein tails, and creation of docking sites for proteins containing bromodomains or chromodomains that recognize acetylated or methylated lysine, respectively (34). The term "histone code" is used to describe the influence of histone modifications on gene expression and indicates that histone machinery decides which part of gene is transcribed (35). Genome-wide studies have revealed that different regions have distinct histone-modifications patterns, enabling expression of specific class of genes. A large number of studies contributed to understanding the functions of individual histone modifications. And thus, acetylation of lysine residues, is believed to be enriched in highly active promoters and increase transcription. Lysine: 4, 36, 79 mono-, di-, tri-methylation and 9, 27 mono-methylation is associated with active genetic status. Conversely, lysine: 9, 27 di- and tri-methylation is a repressive mark, resulting in gene inactivation. In turn, H3ser10P is responsible for gene activation and, like H3ser28P, for chromosome condensation during mitosis (36, 37). Histone modifications can alter as a result of activation process in CD4+ cells. Lamere SA. et al. (38) have revealed, that upon CD4+ activation, the dynamics of H3K4 methylation in promoter varies, and matches the RNA expression. Many differences in histone H3 methylation have been observed in gene promotors between Tregs and Teffs (39). In our study we decided to measure 21 histone H3 modifications, which consisted of lysine: 4, 9, 27, 36, 79 methylation, serine: 10, 28 phosphorylation and lysine: 9, 14, 18, 56 acetylation. We conclude that the type of stimulation (whole insulin or insulin β chain peptide 9-23) has an impact on PTMs.

The process of histone alteration is dynamic upon environmental conditions and is believed to be an indicator of gene activation status (40). The study of Th1 and Th2 differences in histone modifications in crucial gene signatures confirms the presence of active marks in given cell population with repressive histone marks in opposing cell line (41, 42). Moreover, substantial differences were not found between Tregs and conventional T cells based on H3K4me4 and H3K27me3 modifications (39). In our study we also did not see many differences between polyclonal Tregs and Teffs, except H3K79me3, with a predominance in Tregs (**Figure 6A**). However, upon antigen stimulation we observed changes in histone H3 modifications. In general, permissive H3 modifications (H3K4me1, H3K4me3) are abundant in indispensable regions such as: *FoxP3* promoter and intronic enhancer elements, and are connected with active promoters of up-regulated genes (*IL2RA, CTLA4, TNFRSF18, FOLR4*) (28, 43). All the above allow to maintain stable *FoxP3* expression and cell lineage commitment. In our study, Tregs $SPEC_{INS}$ were the least modified of H3K4me1/3, but high amount of such modifications was seen in all Tregs subsets, with the predominance of Tregs $UNSPEC_{INS}$ (**Figure 6A**). Another important modification regarding Tregs is H3 acetylation by histone acetyltransferases (HATs) CBP and p300. It allows proper development and maintenance of the suppressive function of Tregs. Upon activation HATs mediate acetylation of Tregs-related genes permitting their stable function. Disruption of p300 causes Tregs instability and promotes autoimmunity (44). Our research has revealed, that Tregs $SPEC_{9-23}$ were more enriched in lysine 9,14,18,56 acetylation compared to Tregs $SPEC_{INS}$ (**Figure 6B**). Regarding H3K27 methylation, the EZH2 methyltransferase contributes to cell stability and normal function.

It might be connected with the closed chromatin state in genes that are down-regulated by *FoxP3* (45–47). Moreover, EZH2 disruption leads to Tregs impairment and strengthens the anti-tumor immunity (48), which indicates the prominent role of H3K27me3 in Tregs. Here, we saw that Tregs $SPEC_{9-23}$ and Tregs $UNSPEC_{INS}$ have higher % of H3K27me3 modification than Tregs $UNSPEC_{9-23}$ and Tregs $SPEC_{INS}$ (**Figure 6A**).

Interestingly, we found a characteristic pattern related to the kind of stimulation in particular Treg/Teff pairs. Namely, a low level of PTMs in one subset from the pair was always associated with high level of PTMs in the other. This trend was found in the cells specific to insulin where low level of PTMs in Tregs $SPEC_{INS}$ was associated with high level of PTMs in Teffs $SPEC_{INS}$ and high level of PTMs in Tregs $UNSPEC_{INS}$ was associated with low level of PTMs in Teffs $UNSPEC_{INS}$. According to global DNA methylation there is an interdependence between low methylation level and high abundance of histone modifications in Tregs $UNSPEC_{INS}$ and Tregs $SPEC_{9-23}$, and decreased H3 modifications in Treg $SPEC_{INS}$ and Tregs $UNSPEC_{9-23}$ in relation to higher percentage of DNA methylation. But this trend did not occur in Teffs subset. There was no difference in Tregs/Teffs $SPEC_{B:9-23}$ pair and the low level of modifications in Tregs $UNSPEC_{B:9-23}$ was associated with the high one in Teffs $UNSPEC_{B:9-23}$. The PTMs in which the differences were significant overlapped only partially between particular Tregs/Teffs pairs, which suggests that the stimulation with different peptides differently influenced PTMs. Nevertheless, mainly activating PTMs, such as H3K18ac, H3K9me1 and, H3K36me2 were modified in Tregs $SPEC_{B:9-23}$

and Tregs $SPEC_{INS}$ and additionally H3K14ac and H3K27me1 were modified only in Tregs $SPEC_{B:9-23}$. It is also important to note that selectivity index (SI) is almost always below 1 for POLY and $UNSPEC_{INS}$ Teffs/Tregs pairs (Tregs more modified than Teffs) and above 1 in other pairs (Tregs less modified than Teffs) (**Table 4**).

Gene expression analysis confirmed that antigen stimulation did not deprive Tregs of the expression of crucial genes. All Tregs subsets had high expression of genes (*FoxP3, IKZF4, IKZF2, CTLA4, IL2RA*) needed for their function and phenotype maintenance. Tregs $SPEC_{B:9-23}$ were characterized by the highest expression but there was not much difference between Tregs POLY and Tregs $SPEC_{B:9-23}$. On the other hand, the analysis has revealed diminished gene expression in Tregs $SPEC_{INS}$. It is known, that *TNFRSF18* (GITR) and *IKZF4* (Eos) are constitutively expressed by $FoxP3^+$ cells and their expression in FoxP3- cells increases during activation (49, 50). In our study, we noticed that Teffs $SPEC_{INS}$ and $SPEC_{B:9-23}$ had high or moderate expression of GITR and Eos, respectively, which confirms a state of activation upon antigen stimulation in these cells. Despite the high level of *FoxP3* mRNA expression, we did not see a correlation between high percentage of $FoxP3^{high}$ cells, presented by Iwaszkiewicz-Grzes et al. (14), and mRNA level of *FoxP3*. Accordingly, other study also confirmed a modest relationship between protein levels and mRNA *FoxP3* expression, indicating a presence of other mechanisms involved in *FoxP3* expression (51). Bjur et al. (52) discovered that mRNA levels may not correlate with corresponding proteins due to post-transcriptional modifications. They noticed that, upon cell activation, changes in translational activity of specific mRNAs occur. Moreover, a recent study suggested that FoxP3 protein is subject to PTMs, which can alter its function, or even its stability (53).

Besides Foxp3 analysis, on the seventh day in our previous study (14), we performed phenotype test of: IKZF2, CD25 and CTLA-4, and observed high expression of each of them. The data on Helios and IL2RA are already published (14). Our current study has shown high percentage of relative gene expression of *CTLA4* in Tregs compared to Teffs. Nevertheless, Teffs cells had detectable *CTLA4* mRNA. At the protein level on day 7 after cell stimulation, Tregs and Teffs CTLA-4 expression on the cell surface was around 95% and 85% respectively. It is confirmed that CTLA-4 is continuously expressed on Tregs and occurs in T effector cells after activation, with maximum peak in proliferating, dividing cells (54). The discrepancies between low mRNA level at day 12 in Teffs and surface protein abundance can be explained by the CD4 activation model. Following TCR stimulation *CTLA4* mRNA is detected after 1 h with its peak around 24-36 h, and depends on mRNA half-life which is within the range from 4,6 h to 8,9 h, according to cell stimulation (55). Furthermore, Chan V. et al. conducted a study on CD4+ cells and observed an increase of mRNA *CTLA4* after 1 h after stimulation, maintained until 18 hours (56). Worth emphasizing is that CTLA-4 surface expression is modulated by many factors including TCR stimulation strength and depends on other mechanisms like CTLA-4 internalization and recycling (57).

Collectively, our results clearly demonstrate that stimulation with antigen-loaded monocytes presenting whole insulin or insulin β chain peptide 9-23 exerts epigenetic changes in Tregs. The type of stimulation determines the level of alterations in global DNA methylation pattern, and specific methylation of TSDR region as well as histone H3 PTMs. Insulin β chain peptide 9-23 promotes mainly Treg-oriented changes, while the phenotype after whole insulin stimulation was less clear. Hence, the pattern of the epigenetic changes may help finding the peptides that shape exclusively Tregs-mediated suppressive response or Teffs-mediated inflammatory response in future cellular drugs. Our observations indicate that antigen-specific Tregs during cell culture remained stable and comprise all Tregs-related features. It strengthens our confidence that our protocol allowing to obtain antigen-specific Tregs is a promising strategy of cell therapy, e.g. in type 1 diabetes.

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 below: Zenodo and 4442316 doi: 10.5281/zenodo.4442316.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, DI-G, MG and PT. Methodology, DI-G, MP, MG, ZU-W and PT. Software, DI-G. Validation, DI-G and MP. Formal analysis, DI-G, MP and MG. Investigation, DIG and PT. Writing—original draft preparation, DI-G, MP and MG. Writing—review and editing, PT. Visualization, DI-G. Supervision. PT. Project administration, DI-G and PT. Funding acquisition, DI-G and PT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.642678/full#supplementary-material

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Regulatory B Cells Dysregulated T Cell Function in an IL-35-Dependent Way in Patients With Chronic Hepatitis B

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Interleukin (IL)-35-secreting B (IL-35+B) cells are critical regulators in autoimmune and infectious diseases and exert suppressive functions in parallel with IL-10-producing B (B10) cells. However, the role of IL-35+B cells in persistent hepatitis B virus (HBV) infection remains unclear. To elucidate the role of IL-35+B cells in the progress of chronic HBV infection, we determined the frequency of IL-35+B cells and their relationship with the classical human regulatory B cell (Breg) subsets, namely, CD19+CD24^{hi}CD38^{hi} and CD19+CD24^{hi}CD27+. Then, the regulatory effect and mechanism of Bregs on effector T cells were investigated *in vitro*. Here, we found that compared with healthy controls, the frequency of IL-35+B cells was increased in patients with chronic HBV infection and was enriched in human classical Breg subset CD19+CD24^{hi}CD38^{hi} B cells. Moderate correlation was observed between the frequency of IL-35+B cells and alanine aminotransferase levels (Spearman r = 0.401), but only mild correlation was noted between the frequency of IL-35+B cells and HBV DNA level (Spearman r = 0.314). The frequency of IL-35+B cells was negatively correlated with interferon-γ (IFN-γ)-producing CD4+ and CD8+ cells but positively correlated with IL-4-producing T cells. Bregs dysregulated T cell function through an IL-35-dependent mechanism and depended on cell-to-cell contact. In conclusion, IL-35+ B cell was enriched in CD19+CD24hiCD38hi B cell subset during persistent HBV infection and Breg cells exerted dysregulation in T cell function through IL-35 dependent mechanism and depend on cell-to-cell contact.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier NCT03734783.

Keywords: chronic HBV infection, regulatory B cells (Bregs), interleukin 35 (IL-35), IL-35-secreting B (IL-35+B) cells, HBV - hepatitis B virus, immune regulation

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major global health concern that is correlated with the occurrence of liver cirrhosis and hepatocellular carcinoma and affects more than 200 million patients worldwide (1). Host acquired antiviral immunity, especially T cell immunity, is vital in HBV clearance during acute and chronic HBV infection, however, once a chronic HBV infection is established, viral-specific T cell immunity is manifested as functional imbalance or exhaustion, and the mechanism is not yet fully understood (2–6). Immune suppressor cells such as CD4 +CD25^{high}FoxP3+ regulatory T cells (Tregs) (4), costimulatory factors such programmed death factor 1 pathway (7, 8), and Treg/Th17 imbalance (9, 10) are important in the exhaustion of T cell immune function during chronic HBV infection.

Regulatory B cells (Bregs), newly identified regulatory cells, exert immunoregulatory roles in the course of infectious diseases (11, 12), autoimmune diseases (13, 14), and cancer (15, 16) by secreting interleukin (IL)-10, transforming growth factor-β1 (TGF-β1), IL-35, and other inhibitory cytokines. Das et al. (17) studied the role of Bregs in persistent HBV infection and found that IL-10-producing B (B10) cells are enriched in patients with chronic hepatitis B (CHB) and the frequency of B10 cells is correlated temporally with hepatic flares. In another study, Gong et al. (18) simply defined Bregs as CD19+IL-10+ cells and demonstrated that these Bregs have similar immune regulatory function to Tregs during chronic HBV infection.

IL-35 (p35/Ebi3), an anti-inflammatory cytokine of the IL-12 family, which is highly expressed in CHB patients and plays an important role in the inhibition of cellular immune response (19–21), is another major effector cytokine of Bregs. Shen et al. (22) had demonstrated that IL-35-secreting B (IL-35+B) cells are another critical regulator during autoimmune and infectious diseases and exert suppressive functions in parallel with B10 cells. However, the role of IL-35+B cells in CHB remains unclear. In the present study, we aimed (1) to examine the frequency of IL-35+B cells in total peripheral blood B cells of CHB patients, (2) to determine the frequency of IL-35+B cells in two humans classical Breg subsets in CHB, (3) to investigate whether Bregs regulated T cell function through IL-35 pathway, and (4) to explore the possible mechanism of Bregs on effector T cells.

MATERIALS AND METHODS

Patients and Controls

All individuals were recruited according to the protocol that was approved by the clinical Ethics Committees of Shanghai Changhai Hospital (CHEC2017-118) and registered on *Clinicaltrials.gov* (NCT03734783). The study was conducted between July 2017 and November 2019. Written informed consent was obtained from all participants.

Patients with CHB were all sero-positive for hepatitis B surface antigen (HBsAg) for at least 6 months and negative for other hepatotropic viruses, such as hepatitis D virus (HDV), hepatitis C virus (HCV), hepatitis E virus, hepatitis A virus, and human immunodeficiency virus 1/2. Healthy controls (HCs) did not have any previous history and current evidence of liver disease. Serum alanine aminotransferase (ALT) values were normal, and HBsAg and other hepatitis virus markers were negative. Furthermore, all participants were sero-negative for markers such as ceruloplasmin, anti-nuclear antibodies and anti-mitochondrial antibodies for co-existent autoimmune and metabolic liver diseases.

Direct Labeling and Intracellular Labeling Flow Cytometric Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (Ficoll-Hypaque density gradient, Axis-shield, Germany). The antibodies and fluorochromes used in this work are shown in **Table S1**.

For Breg subset and B10 or IL-35+B cell detection, PBMCs (2×10^6) were stimulated with CD40L (5 μg/mL, Purified Anti-Human CD154 (CD40L), Tonbo Biosciences, USA) plus CpG-ODN (1.5 μM, TLR9 Agonist-Stimulatory Class B tlrl-2006, InvivoGen) and lipopolysaccharide (LPS, 1 μg/mL, eBioscience) for 48 h. Furthermore, cells were activated for another 4 h at 37°C in 5% CO_2 with 50 ng/mL phorbol myristate acetate, 1 mmol/L ionomycin (both from Sigma, St. Louis, MO, USA), and 10 mg/mL brefeldin A (Tocris Cookson, Bristol, UK) in complete RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA). The cells were then collected and washed with PBS once and stained for surface markers. Then, they were permeabilized with Perm/Fix solution (Cytofix/Cytoperm^{TM}, BD biosciences) and stained intracellularly with specific fluorescent conjugated anti-human IL-10, eBi3/IL-12/IL-35p35 (IL-35). For B cell subsets, we analyzed two classical Breg subsets: CD24^{hi}CD38^{hi}, CD24^{hi}CD27+, the total IL-10 secreting B cells and IL-35 secreting B cells, which were all gated on CD19+ cells (**Figure S1B**). We also determined the frequency of IL-35 producing Breg: CD24^{hi}CD38^{hi}IL-35+, CD24^{hi}CD27+IL-35+, and IL-10 secreting Breg: CD24^{hi}CD38^{hi}IL-10+, CD24^{hi}CD27+IL-10+, frequency was determined into CD24^{hi}CD38^{hi} and CD24^{hi}CD27+ gates, respectively (**Figure S1**).

For T cell subsets detection, freshly isolated PBMCs (2×10^6) were stimulated for 5 h with 50 ng/mL phorbol myristate acetate,1 mmol/L ionomycin (both from Sigma, St. Louis, MO, USA), and 10 mg/mL brefeldin A (Tocris Cookson, Bristol, UK) in complete RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA). Upon harvest, cells were first stained with surface markers (CD3-APC-Cy7-A, CD4-FITC-A, and CD8-PerCP-Cy5-5-A) and then intracellularly stained with IL-17-APC-A, IFN-γPE-Cy7-A, and IL-4-PE-A. All these T cell subsets were gated on PBMC cells (**Figure S2**).

Flow cytometry was performed using a FACSCalibur (Becton Dickinson, San Jose, CA). FACS data were analyzed using CellQuest software (Becton Dickinson Rutherford, NJ).

Luminex Multiplex Cytokine Assays

Serum concentrations of inflammatory cytokines, including IL-4, IL-17A, IL-21, and interferon-γ (IFN-γ), were measured using

commercially available Luminex MAP kits (ProcartaPlex 5 plex, 1 plate 5-plex, eBioscience) in accordance with the manufacturer's instructions. Samples were two times diluted with 1×universal assay buffer and tested in triplicate.

Enzyme-Linked Immunosorbent Assay

Serum and PBMC culture supernatant concentrations of IL-35 and IL-10 were measured by using commercially available ELISA kits (XpressBio, USA; Proteintech, USA) in accordance with the manufacturers' instructions. All samples were not diluted and assessed in triplicate.

Suppression Effect on T Cells

Purified B cells isolated from PBMC using anti-human CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were treated with CpG/CD40L/LPS with or without HBVcore (1-183 a.a) (ProSpec, USA) for 48 h. After stimulation, B cells were washed with PBS twice and then co-cultured with autologous CD19-depleted PBMCs (50,000 cells) at the ratio of 2:1 in anti-CD3/CD28 (working concentration of anti-CD3 and anti-CD28 was 10μg/ml and 2μg/ml respectively, Tonbo Biosciences, San Diego, CA)-coated 96-well plate in complete RPMI-1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% heat-inactivated fetal bovine serum (Gibco,Grand Island, NY, United States) for another 48 h. After 48 h of co-culture, the frequency of IL-4-, IFN-γ-, and IL-17A-secreting T cells was evaluated by flow cytometry.

Transwell Assay and Blocking Study

Transwells of 0.4 μm pore size were used (Millicell, Merck Millipore, Billerica, MA, USA). CD19-depleted PBMCs (50,000 cells) were added to the lower chambers of 24-well plates and subsequently stimulated with anti-CD3/anti-CD28 as described above, whereas non-treated or stimulated B cells (100,000) were added to the upper chambers in the transwells. As controls for the transwell assay, CD19-depleted PBMCs (50,000 cells) and stimulated B cells (100,000 cells) were also co-cultured in the same plate in the lower chambers with anti-CD3/anti-CD28. After 48 h of co-culture, CD19-depleted PBMCs were gathered, and the frequency of IFN-γ-secreting T cells was tested by flow cytometry. For cytokine blocking study, anti-IL-12/IL-35 p35 (1 μg/mL, R&D Systems) and anti-IL-10 (5 μg/mL, Invitrogen) were added to the co-culture system to investigate the potential mechanism of Breg on T cells.

HBV DNA Quantification

Serum HBV DNA levels were quantified using fluorescent quantitative PCR with commercially available kits (Sansure Biotech, China). The detection range was 1×10^2 to 5×10^8 IU/mL.

Viral Serological Test

The levels of HBsAg, HBeAg, anti-HBs, anti-HBc, anti-HBe, anti-HCV, anti-HDV, anti-HGV, anti-HIV-1, and anti-HIV-2 were measured using commercially available kits (Abbott Laboratories, North Chicago, IL) in our clinical laboratory. The dynamic range of serum HBsAg was 0.05–250 IU/mL. The samples were diluted to 1:500 or 1:1000 using the ARCHITECT HBsAg Manual Diluent (Abbott Diagnostics) if >250 IU/mL.

Statistical Analysis

Normally distributed continuous quantitative data were expressed as the mean ± SEM, and non-normally distributed continuous quantitative data were expressed as median (interquartile range, IQR). The non-parametric Mann–Whitney U test was used to evaluate the differences between two independent samples, and the non-parametric Kruskal–Wallis ANOVA test was used to evaluate the differences in more than two groups. The non-parametric Friedman ANOVA test was used to evaluate effector T cell proportions in co-culture experiments. The statistical correlation between variables was calculated by Spearman rank correlation analysis. P-value < 0.05 was considered statistically significant. All analyses were performed using SPSS software (version 21.0.0; Chicago, IL). The graph was made using GraphPad Prism 5 (San Diego, CA, USA).

RESULTS

Patients' Clinical Characteristics

The clinical and biochemical characteristics of the studied patients are listed in **Table 1**. All 68 CHB patients were treatment-naïve at enrollment. Approximately 57.35% (39/68) of patients had ALT levels greater than the upper limit of normal (ULN) at enrollment, and higher proportions of patients in the abnormal ALT group were HBeAg positive compared with those in the normal ALT group. Similarly, HBeAg-positive patients at enrollment had higher median ALT levels than HBeAg-negative patients. Twenty-six age- and gender-matched HCs were enrolled.

Serum IL-35 Levels Were Correlated With Liver Flare and Viral Replication

To determine the role of IL-35 in persistent HBV infection, we first determined the serum IL-35 concentration in 68 patients with persistent HBV infection. Compared with HCs, CHB patients had significantly increased serum IL-35 levels. Stratified analysis found that the serum IL-35 levels were significantly increased in 39 patients with liver flare (**Figure 1A**); patients with active viral replication, including 39 HBeAg+ CHB patients; and 49 patients with viral load more than 2E4 IU/mL (**Figures 1A, B**).

IL-35+B Cells Were Enriched During Persistent HBV Infection and Correlated With Liver Injury

Breg response was defined by functional cytokines (23), and many researches have demonstrated that IL-35+B cells play a suppressive regulatory role in autoimmune and infectious diseases (22–24). However, the role of IL-35+B cells in chronic HBV infection is still unclear. To evaluate the role of circulating mononuclear cells in IL-35 secretion in persistent HBV infection, we quantified the frequency of IL-35+B cells in total B cells in 35 patients with chronic HBV infection. Compared with that in

TABLE 1 | Individuals demographic and clinical characteristics.

Characteristics	HCs	CHB	ALT nor.	ALT abnor.	HBeAg pos.	HBeAg neg.	P* value	P# value	P$ value
Number	26	68	29	39	39	29	/	/	
Age (years)	33.24 ± 8.99	36.84 ± 8.67	34.00 (29.50,42.50)	35.00 (29.00,41.75)	34.00 (28.75,41.25)	36.00 (31.00,43.00)	0.12	0.59	0.09
Sex (M:F)	16:10	50:18	17:12	33:6	29:10	21:8	0.38	0.03	1.00
HBeAg positive, n(%)	/	40(58.82)	9(31.03)	30(76.92)	/	/	/	<0.001	/
HBsAg, Log_{10} (U/L)	/	3.74 ± 0.74	3.55 ± 0.80	3.75 ± 0.68	4.03 ± 0.53	3.21 ± 0.53	/	0.27	<0.001
HBV DNA, Log_{10} (IU/ml)	/	4.15(2.90,7.79)	2.50(1.70,7.33)	6.47(4.68,7.82)	7.73(6.36,7.90)	2.50(1.70,4.15)	/	0.001	<0.001
ALT,IU/L	26.00 (18.00,26.50)	37.00 (19.50,237.50)	23.00 (15.50,33.50)	191.50 (120.25,365.00	152.50 (64.75,349.00	149.00 (33.00,239.40	<0.001	<0.001	0.001
AST,IU/L	18.00 (16.00,26.00)	31.00 (17.50,88.50)	18.00 (16.50,22.00)	80.50 (48.25,163.25)	73.00 (36.50,161.75)	44.00 (21.00,101.60)	<0.001	<0.001	<0.001
WBC,×10^9/L	4.98(4.98,5.29)	5.28 ± 1.19	5.71 ± 1.06	5.30 ± 1.36	5.185.30 ± 1.28	5.84 ± 1.15	0.06	0.25	0.05

HCs, healthy controls; CHB, chronic hepatitis B; ALT, Alanine aminotransferase; AST, aspartateaminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; WBC, white blood cells; nor., normal; abnor., abnormal; pos., positive; neg., negative; P, CHB Vs HC; P#, ALT nor. Vs ALT abnor. ALT; P$, HBeAg pos. Vs BeAg neg.*

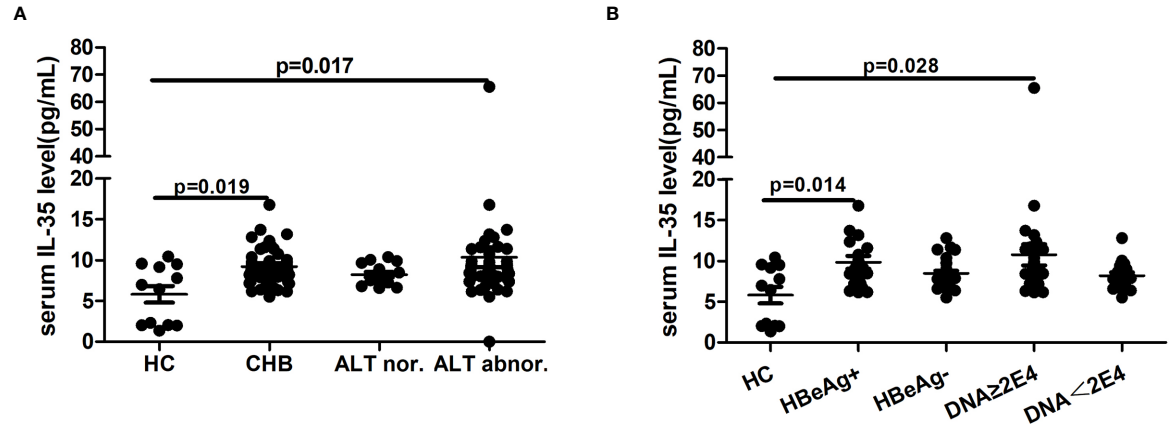

FIGURE 1 | Serum IL-35 level in patients with chronic hepatitis B. 68 CHB patients were enrolled in this assay. Non-parametric Mann-Whitney U and Non-parametric Kruskal-Wallis ANOVA test were used to evaluate the differences between two groups and among groups more than two, respectively. P value<0.05 was considered statistically significant. **(A)** Serum IL-35 level in patients with different degree liver inflammation; **(B)** Serum IL-35 level in patients with different viral replication level. Data are shown as mean ± s.e.m.

HCs, the frequency of both IL-35+B cells and IL-10-secreting B (B10) cells in total B cells in patients with chronic HBV infection was not increased significantly. (**Figure 2A**).

To analyze the relationship between IL-35+B cells and liver inflammation degree and viral replication level, we divided the treatment-naïve patients into two groups according to the ALT levels: patients with high ALT levels (ALT ≥ 200 U/L, n=25) and patients with low ALT levels (ALT < 200 U/L, n=10). Then, we also divided the patients into two groups according to HBeAg status and HBV DNA levels: patients with high virus replication (HBeAg positive, n=19; or HBV DNA ≥ 2E4 IU/mL, n=17) and patients with low virus replication (HBeAg negative, n=16 or HBV DNA < 2E4 IU/mL, n=18). Compared with HCs and patients with low ALT levels, patients with high ALT levels had the highest frequency of IL-35+B and B10 cells (**Figure 2B**). Among patients with different HBeAg status or viral loads and HCs, no significant difference was found in the frequency of IL-35+B cells (**Figures 2C, D**) in the peripheral blood total B cells. However, in patients with low viral replication, the frequency of B10 cells was higher than that of IL-35+B cells (**Figures 2C, D**). According to Spearman correlation analysis, a moderate correlation was observed between the frequency of IL-35+B cells and ALT levels (**Figure 2E**), but only mild correlation was found between the frequency of IL-35+B cells and HBV DNA levels (**Figure 2F**). The frequency of B10 cells was only moderately correlated with serum ALT levels in these patients (**Figure S3**).

Increased Frequency of IL-35+B Cells Was Correlated With Classical Breg Subsets in Patients With Chronic HBV Infection

To quantify the frequency of various Breg subsets, the PBMCs of 33 CHB patients (17 HBeAg-positive patients and 16 patients in the abnormal ALT group) and 11 HCs were isolated and stimulated with CpG plus CD40L and LPS *in vitro* for 48 h,

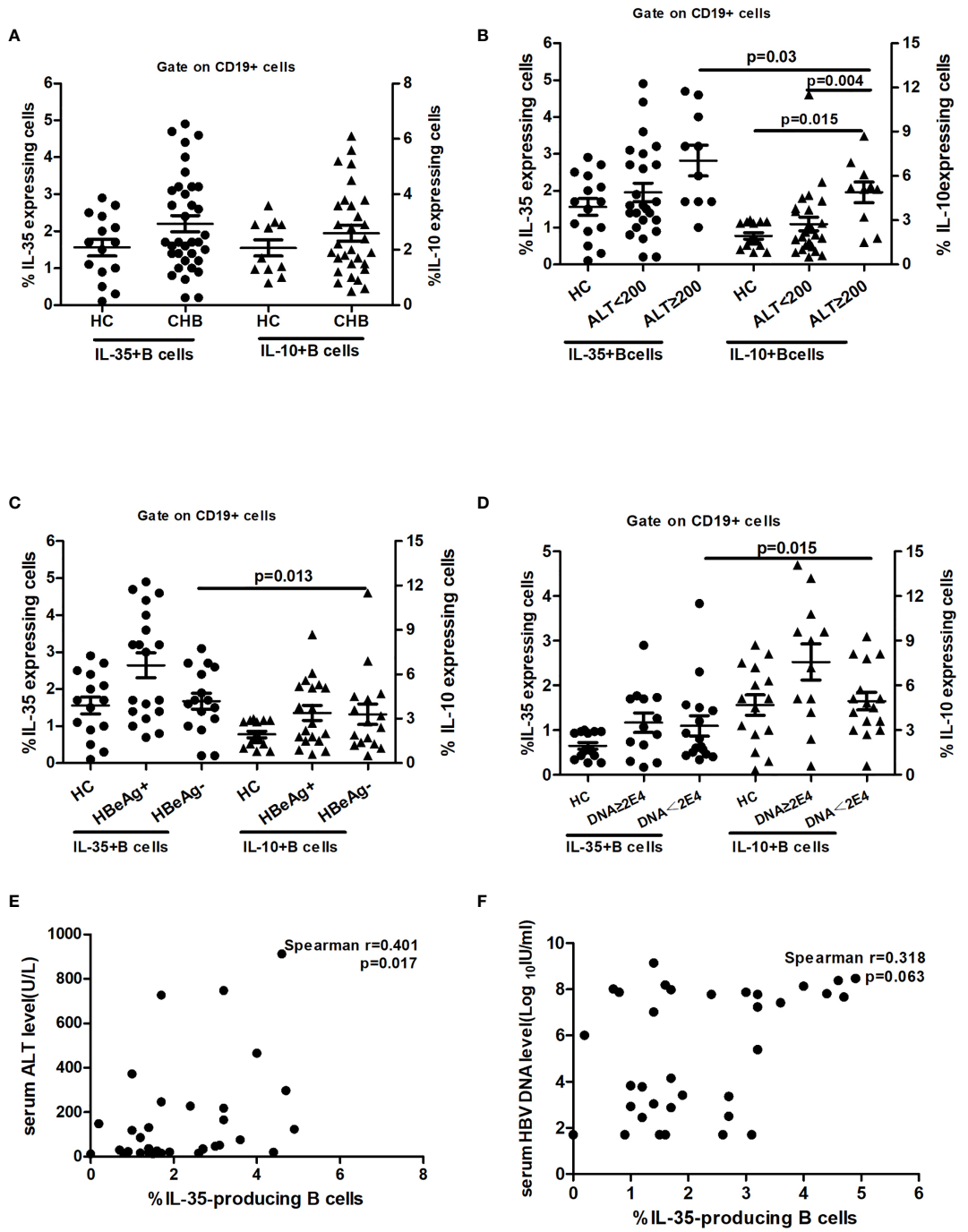

FIGURE 2 | IL-35-producing B cells in patients with chronic hepatitis B. 35 CHB patients (25 of them with ALT ≤200, 17 of them with HBeAg positive and 13 of them with HBV DNA≥2E4) and 15 HCs were enrolled in this assay. Difference between two groups and among groups more than two was test by Non-parametric Mann-Whitney U test and Non-parametric Kruskal-Wallis ANOVA test, respectively. Statistical correlation between variables was calculated by the Spearman rank correlation analysis. P value<0.05 was considered statistically significant. **(A)** IL-35-producing B cell was enriched and in parallel with IL-10-producing B cells in patients with chronic hepatitis B; **(B)** Patients with chronic HBV infection had high frequency of IL-35+B and IL-10-producing B cells cells in the peripheral blood; **(C)** Frequency of IL-35-producing B cells and IL-10-producing B cells in patients with different HBeAg status; **(D)** Frequency of IL-35-producing B cells and IL-10-producing B cells in patients with different viral load; **(E)** IL-35-producing B cells were moderately correlated with ALT level; **(F)** IL-35-producing B cells were mildly correlated with serum viral load.

which was proved to potently induce Breg differentiation (22, 25). Then, the frequency of classical human Breg subsets CD19+CD24^{hi}CD38^{hi} and CD19+CD24^{hi}CD27+ was quantified using flow cytometry. Compared with HCs, the frequency of CD19+CD24^{hi}CD38^{hi} (19.76% ± 3.64% vs. 8.43% ± 3.46%) and CD19+CD24^{hi}CD27+ (11.42% ± 2.37% vs. 6.28% ± 2.18%) increased in patients with CHB (**Figure 3A**). Further stratified analysis of the frequency of Breg subsets in patients according to HBV replication and liver inflammation degree showed that patients with active liver inflammation (ALT ≥ 2*ULN) and virus replication (HBeAg positive) had higher frequency of Breg subsets, and in these patients, the Breg subset was dominated by CD19+CD24^{hi}CD38^{hi} (**Figures 3B, C**).

As IL-35 is one of the markers of phenotypic changes in B cells, we analyzed the relationship of the frequency of IL-35+B cells with human classical Breg subsets during persistent HBV infection. As shown in **Figures 3D, E**, the frequency of IL-35+B cells was strongly correlated with that of two classical human Breg subsets during persistent HBV infection (Spearman r = 0.749 and 0.782, P < 0.001). Furthermore, we found that patients with persistent HBV infection had higher frequency of IL-35-positive Breg subsets compared with HCs, but no difference was observed between the two classical Bregs (**Figure 3F**).

Correlation of IL-35+B Cells and Th1/Th2 Balance in CHB Patients

The main function of Bregs is to regulate the differentiation of Th cells and exert suppressive function on T cell proliferation through their effector factors, such as IL-10, IL-35, and TGF-β1. To evaluate the function of IL-35+B cells during persistent HBV infection, the frequency of IL-35+B cells and homologous effector T cell subsets was measured, and the correlation of IL-35+B cells with various T cell subtypes in CHB patients was analyzed using Spearman's rank correlation analysis. We found that the frequency of IL-35+B cells was negatively correlated with that of IFN-γ-producing CD4+ and CD8+ cells, but positively correlated with that of IL-4-producing T cells (**Table 2**). However, a weaker correlation was found between serum IL-35 levels and different T cell effector cytokines (**Table S2**).

Suppression Effect on Effector Cells

To determine the suppressive effect of Bregs on T cells, B cells isolated from PBMCs using anti-CD19 microbeads were stimulated with CpG/CD40L/LPS with or without HBVcore peptide for two days. Then, autologous CD19-depleted PBMCs were co-cultured with HBV-exposed B cells or stimulated B cells and subsequently activated with anti-CD3/anti-CD28 for another 48 h. Because of the importance of Th1/Th2 imbalance in CHB disease progression, we aimed to determine the regulatory effect of Bregs on IL-4- and IFN-γ-producing T cells. As shown in **Figures 4A–E**, ECs were stimulated significantly *in vitro* by anti-CD3/CD28, and Bregs significantly decreased the frequency of IFN-γ-producing CD4+ and CD8+ T cells (**Figures 4A, D**). However, the frequency of IL-4-producing T cells and IL-17-producing T cells were not significantly affected by Bregs (**Figures 4B, C, E**).

Bregs Play Immunoregulatory Roles by Secreting IL-35 During Persistent HBV Infection

Das et al. (17) had shown that Bregs exert their immunoregulatory role by secreting IL-10 during persistent HBV infection. We aimed to determine whether the IL-35 pathway is also involved in Bregs' suppressive function during persistent HBV infection. As shown in **Figure 5A**, IL-35 neutralizing antibody could significantly reverse the suppressive effect of Bregs on EC cytokine production, but no synergistic function of IL-35 and IL-10 neutralizing antibody was found. Furthermore, we found that the suppressive function of Bregs on ECs was significantly decreased in the trans-well co-culture system (**Figure 5B**).

DISCUSSION

Bregs, especially B10 cells, are involved in the process of chronic HBV infection, such as CHB (17, 18, 26, 27), HBV-related liver cirrhosis (28), and chronic HBV infection-related renal injury (29) *via* an IL-10-dependent manner. These findings have prompted studies into the mechanism of B-cell-mediated immune regulation, particularly the suppressive cytokines secreted by B cells. During the process of chronic HBV infection, changes in serum IL-35 levels were observed compared with HCs (21, 30). Shi YY et. al (30). found that in the process of persistent HBV infection, the serum IL-35 levels increased gradually along with disease progression and were positively correlated with the liver inflammation degree. However, Cheng et al. (21) found that serum IL-35 levels were significantly decreased in patients with chronic HBV infection and negatively correlated with serum HBV DNA load and ALT levels. In our previous study (31) and the current research, we confirmed that the serum IL-35 levels in treatment-naïve chronic HBV patients were significantly increased compared with those in HCs, and patients with higher ALT levels (>200 or 300 IU/L) or HBeAg-positive patients had higher serum IL-35 levels. Our findings are consistent with those of Shi et al., suggesting that IL-35 exerts an anti-inflammatory effect during persistent HBV infection. However, the difference between the findings of Cheng et al. (21) and ours suggested that more research is needed to determine the expression and role of IL-35 at different stages of HBV infection.

The role of Bregs in chronic HBV infection was identified by Das et al. (17) in 2012. Since then, several studies have investigated the role of Bregs in chronic HBV infection and HBV infection-related mergers (18, 26, 28, 29). However, these studies only focused on the role of B10 cells. In the present study, we confirmed for the first time that IL-35+B cells were enriched and in parallel with B10 cells in patients with persistent HBV infection. Similar to B10 cells, the frequency of IL-35+B cells was associated with liver inflammation and HBV replication level. These findings suggested that in the process of persistent HBV infection, B cells may exert immune regulation through different pathways. Furthermore, we also confirmed that two typical

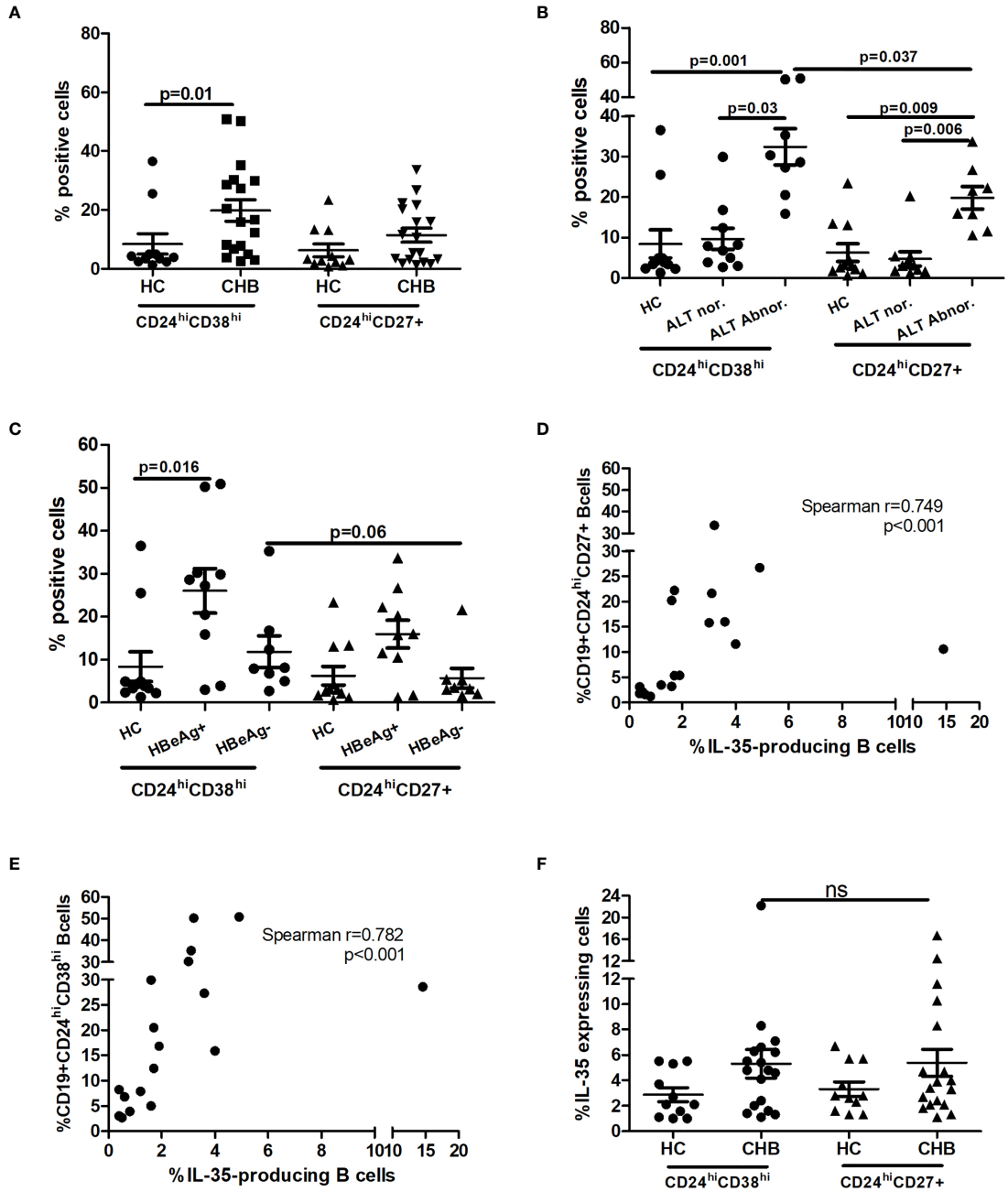

FIGURE 3 | Breg subsets in CHB patients and healthy controls (HCs). Two human classical Breg subsets was gated on CD19+ cell. **(A)** Frequency of CD19+CD24^{hi}CD38^{hi} or CD19+ CD24^{hi}CD27+ Breg subsets in total CHB patients and HCs; **(B)** Frequency of CD19+CD24^{hi}CD38^{hi} or CD19+ CD24^{hi}CD27+ Breg subset in CHB patients according to different liver inflammation; **(C)** Frequency of CD19+CD24^{hi}CD38^{hi} or CD19+ CD24^{hi}CD27+ Breg subsets in CHB patients according to different virus replication level; **(D)** The frequency of IL-35-producing B cells was strongly correlated with the frequency of CD19+ CD24^{hi}CD27+ Breg subset; **(E)** The frequency of IL-35-producing B cells was strongly correlated with the frequency of CD19+CD24^{hi}CD38^{hi} Breg subset; **(F)** Higher frequency of IL-35-positive Breg subset in patients with chronic HBV infection. Difference between two groups and among groups more than two was test by Non-parametric Mann-Whitney U test and Non-parametric Kruskal-Wallis ANOVA test, respectively. Statistical correlation between variables was calculated by the Spearman rank correlation analysis. P value<0.05 was considered statistically significant. ns, no significant difference.

human Breg subsets (CD19+CD24^{hi}CD38^{hi} and CD19+CD24^{hi}CD27+) were enriched and strongly correlated with the frequency of IL-35+B cells. These results further demonstrated that IL-35+B cells mainly belong to Breg subsets.

Previous studies had demonstrated that Bregs are enriched and exert a regulatory role on multiple immune ECs including T cells in CHB patients (17, 27, 32). These studies showed that Bregs play a regulatory role on HBV-specific T cell responses in an IL-10-

TABLE 2 | Correlation between frequency of IL-35-producting B cells or Bregs with various T cell subsets.

Characteristics		Chronic HBV infection	
Condition (1)	**Condition (2)**	**Coefficient**	**P value**
%IL-35-producing B cells	**%IL-4+CD4+**	0.351	0.093
	%IFN-γ+CD4+	-0.073	0.693
	%IL-17A+CD4+	0.062	0.772
	%IL-4+CD8+	0.260	0.151
	%IFN-γ+CD8+	-0.399	0.024
%CD19+CD24^{hi}CD38^{hi}	**%IL-4+CD4+**	.298	0.230
	%IFN-γ+CD4+	0.104	0.681
	%IL-17A+CD4+	0.067	0.790
	%IL-4+CD8+	0.516	0.028
	%IFN-γ+CD8+	0.286	0.250
%CD19+CD24^{hi}CD27+	**%IL-4+CD4+**	0.364	0.137
	%IFN-γ+CD4+	0.042	0.868
	%IL-17A+CD4+	0.086	0.734
	%IL-4+CD8+	0.628	0.005
	%IFN-γ+CD8+	0.276	0.268

In bold: %IL-35-producing B cells: Percent of IL-35-producing B cells on total B cells; %CD19+CD24hiCD38hi: percent of CD24hiCD38hi B cells on total B cells; % CD19+CD24hiCD27+: percent of CD24hiCD27+B cells on total B cells; %IL-4+CD4+: percent of IL-4 positive CD4+T cells on total CD4+T cells; %IFN-g+CD4+: percent of IFN-g positive CD4+T cells on total CD4+T cells; %IL-17A+CD4+: percent of IL-17A positive CD4+T cells on total CD4+T cells; %IL-4+CD8+: percent of IL-4 positive CD8+T cells on total CD8+T cells; %IFN-g+CD8+: percent of IFN-g positive CD8+T cells on total CD8+T cells.

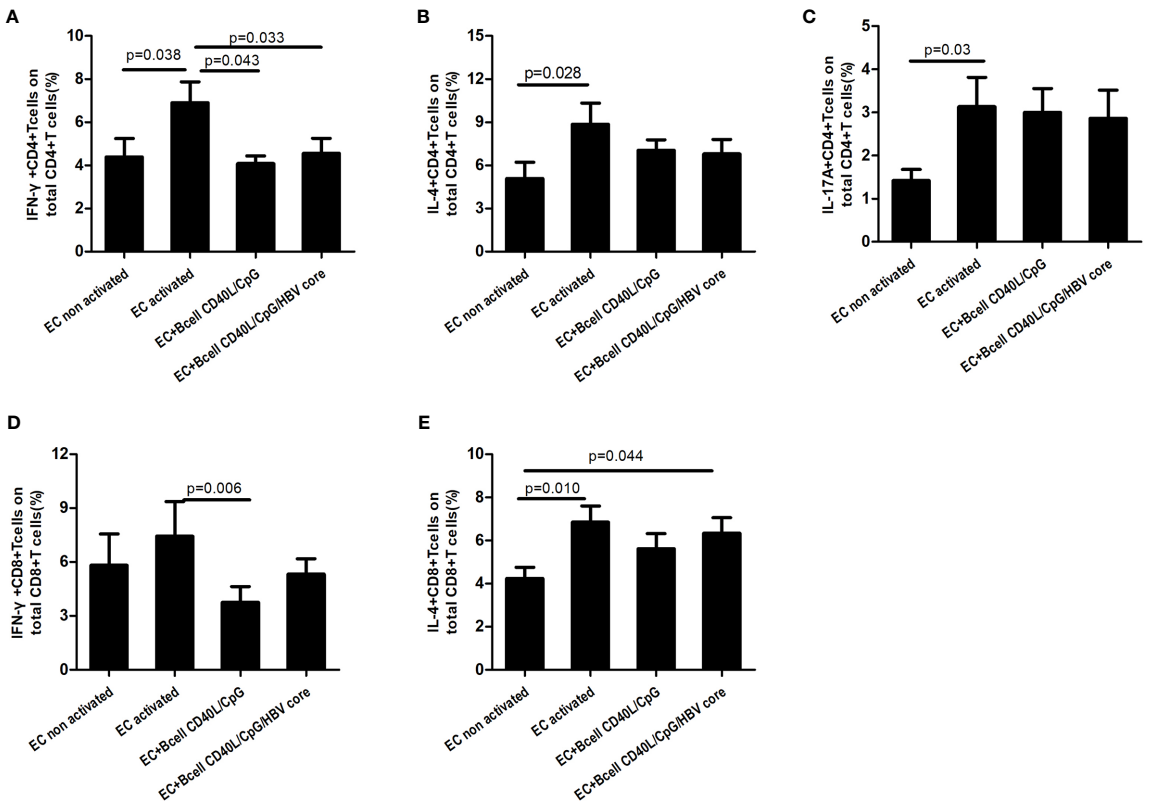

FIGURE 4 | Suppression effect on effector cells (ECs). B cells were isolated from PBMC using CD19+ microbead and stimulated with CD40L/CpG/LPS with or without HBVcore peptide. Stimulated B cells were then co-cultured with autologous CD19-depleted PBMCs at the ratio of 2:1 in anti-CD3/CD28 coated plate for 48hours. As a control, CD19-depleted PBMCs were also cultured alone without B cells (CD19-depleted PBMCs non-activated and CD19-depleted PBMCs activated). **(A)** Suppression effect on IFN-ɤ-producing CD4+T cells; **(B)** Suppression on IL-4-producing CD4+T cells; **(C)** Suppression effect on IL-17A-producing CD4+T cells; **(D)** Suppression on IFN-ɤ-producing CD8+T cells; **(E)** Suppression on IL-4-producing CD8+T cells. Non-parametric Kruskal-Wallis ANOVA test and Tukey's Multiple Comparison test was used to analysis the data. P value<;0.05 was considered statistically significant.

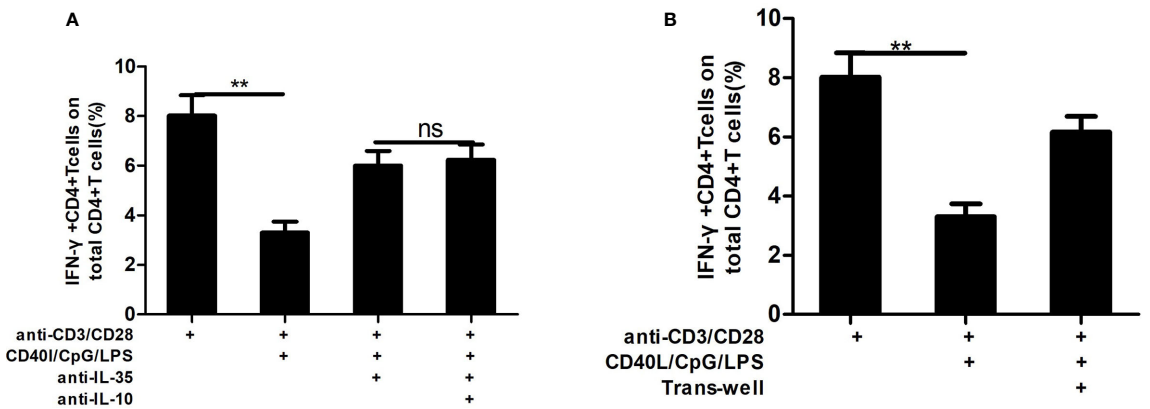

FIGURE 5 | IL-35 neutralizing antibody blocking study and trans-well assay. To explore whether IL-35 worked alone or in collaboration with IL-10 in the process of Bregs regulating on T cell function, neutralizing antibody against IL-35 (1μg/ml, R&D Systems) alone or with neutralizing antibody against IL-10 (5 μg/ml, invitrogen) was added in the co-culture system of anti-CD3/anti-CD28 activated CD19-depleted PBMCs and Bregs. **(A)** Neutralizing antibody against IL-35 can block suppression function of Bregs on CD4+T cytokine secreting function; **(B)** Bregs suppressed CD4+T cell cytokine secreting depending on cell-to-cell contact. Non-parametric Kruskal-Wallis ANOVA test and Tukey's Multiple Comparison test was used to analysis the data. P value<0.05 was considered statistically significant. **p < 0.01. ns, no significant difference.

dependent manner by inhibiting TNF-α and IFN-γ production and enhancing Treg function. In the present study, we confirmed that Bregs can inhibit IFN-γ production but has little effect on IL-4 production. The inhibitory effect of Bregs on IFN-γ production could be reversed by IL-35 neutralizing antibody *in vitro*, and the inhibitory effect was dependent on cell-to-cell contact. These results suggest that during HBV infection, Bregs participate in the T cell imbalance regulation process through an IL-35-dependent manner. To our knowledge, this is the first time to determine that that during persistent HBV infection, Bregs exert immune regulation through a channel other than IL-10-dependent pathway. However, in other chronic virus infections, such as HIV infection, Lopez-Abente et al. (33) had confirmed that HIV-1 could induce B cell toward the Breg phenotype and express IL-10, TGF-β, and EBI3 or IL-12(p35) mRNA.

In conclusion, during persistent HBV infection, serum IL-35+B cells exited and was enriched in CD19+CD24^{hi}CD38^{hi} B cell subset. Bregs dysregulated T cell function through an IL-35-dependent mechanism, which depended on cell-to-cell contact.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Ethics Committees of Shanghai Changhai Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YYL, YL, TZ, and MJ were involved in recruiting patients, collecting data, and performing a series of *in vitro* experiments. ZFT and GST assisted the intracellular labeling flow cytometric analysis, and data analysis. YYL, MJ and XSL involved in the study concept and design, and XSL obtained funding. XSL performed the data analysis and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.653198/full#supplementary-material

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MINI REVIEW
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Antigen-Specific Regulatory T Cell Therapy in Autoimmune Diseases and Transplantation

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Regulatory T (Treg) cells are a heterogenous population of immunosuppressive T cells whose therapeutic potential for the treatment of autoimmune diseases and graft rejection is currently being explored. While clinical trial results thus far support the safety and efficacy of adoptive therapies using polyclonal Treg cells, some studies suggest that antigen-specific Treg cells are more potent in regulating and improving immune tolerance in a disease-specific manner. Hence, several approaches to generate and/or expand antigen-specific Treg cells *in vitro* or *in vivo* are currently under investigation. However, antigen-specific Treg cell therapies face additional challenges that require further consideration, including the identification of disease-relevant antigens as well as the *in vivo* stability and migratory behavior of Treg cells following transfer. In this review, we discuss these approaches and the potential limitations and describe prospective strategies to enhance the efficacy of antigen-specific Treg cell treatments in autoimmunity and transplantation.

Keywords: regulatory T cells, Tregs, antigen-specific Tregs, autoimmune disease (AD), therapy, transplantation

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INTRODUCTION

Treg cells play an essential role in the maintenance of immune homeostasis by inhibiting pathological responses towards self-antigens and controlling potentially harmful inflammatory reactions following infections. While different T cell populations with immunosuppressive capacity have been described in recent years including type 1 regulatory T (Tr1) cells and T helper 3 (Th3) cells (1, 2), $CD4^+CD127^-CD25^{high}$ T cells that express the transcription factor forkhead box P3 (FOXP3) remain the most studied Treg subset to date and thus, will be the main focus of this review. In healthy individuals, $FOXP3^+$ Treg cells are generated both in the thymus (tTreg) upon intermediate avidity interaction of developing thymocytes with self-peptides (3) and in the periphery (pTreg) during antigen encounter of conventional naïve $CD4^+$ T cells in tolerogenic environments, such as the presence of transforming growth factor beta (TGF-ß) and interleukin-2 (IL-2) (4, 5). Although specific biomarkers that allow the distinction between tTreg and pTreg cells are currently not available, it is assumed that the antigen specificities of these Treg subsets differ substantially due to their distinct developmental origin (6, 7). The T cell receptor (TCR) repertoire of tTreg cells is skewed toward autoantigen recognition and hence, they predominantly maintain self-tolerance by preventing immune responses against the body's own tissues and organs (8). In contrast, pTreg cells mainly recognize non-self-antigens derived from commensal bacteria, infectious pathogens or ingested food and thus, sustain mucosal tolerance,

inhibit inflammation-induced tissue damage and avert allergic reactions (5, 9–11). Importantly, various critical questions about the maintenance and function of these antigen-specific Treg cells remain unanswered, involving their *in vivo* cellular targets, the molecular pathways triggering their activation and the underlying mechanisms controlling their suppressive function.

Considering the crucial functions of FOXP3$^+$ Treg cells in maintaining a healthy state, it is not surprising that defects in their biology can lead to detrimental disruptions of immune homeostasis. In particular, multiple preclinical and human studies have demonstrated that a number of Treg-specific defects are associated with the development of several autoimmune disorders (AID) such as type 1 diabetes (T1D) (12–14), rheumatoid arthritis (RA) (15, 16), multiple sclerosis (MS) (17–19), systemic lupus erythematosus (SLE) (20) and psoriasis (21, 22). These Treg-specific defects include reduced proliferative and migratory capabilities (21, 23) as well as lower expression levels of essential Treg markers, including FOXP3 and CD25 (24–27). Moreover, Treg cells isolated from patients with several AID exhibit impaired immunosuppressive functions associated with reduced expression of anti-inflammatory molecules such as IL-10, cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain-containing 3 (Tim-3) and indoleamine 2,3-dioxygenase (IDO) (16, 28–30), and increased production of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and IL-17 (13, 19). Some studies indicate that these deficiencies are predominantly observed in the naïve Treg compartment which is presumed to be largely comprised of tTreg cells (18, 31). Nonetheless, it is still unclear whether defects of Treg cell numbers and/or function in human AID are limited to disease-associated antigen-specific Treg cells or affect polyclonal Treg populations since the antigen specificity of impaired Treg cells remains insufficiently characterized.

POLYCLONAL VS. ANTIGEN-SPECIFIC TREG THERAPIES

While new key factors and mechanisms underlying Treg biology continue being elucidated, Treg cell-based therapies have been proposed to be a promising strategy for the re-establishment of immune tolerance in individuals with AID, allergies or organ transplantation (32–35). These treatments currently involve either the adoptive transfer of *in vitro* expanded Treg cells, or the administration of immunomodulatory interventions that promote the expansion and/or function of Treg cells *in vivo* (**Figure 1**). Notably, both of these applications have the potential to promote Treg-mediated immune regulation in a polyclonal or antigen-specific manner with each harboring their own advantages and limitations.

The success of adoptive Treg cell therapies depends on multiple critical factors, including the optimal source of Treg cells, appropriate cell isolation and expansion procedures as well as optimal cell dose and number of infusions administered. First early-phase clinical trials investigating the safety of autologous or allogeneic Treg transfer demonstrated good toxicity profiles in patients with T1D (32, 36–38), MS (39), Crohn's disease (33), graft versus host disease (GvHD) (40–42) and kidney/liver transplantation (34, 43–46). In addition, some of these interventions induced signs of disease improvement which supported the investigation of treatment efficacy in larger trials (36–38, 41, 46). Importantly, while these initial human studies adopted somewhat comparable cell enrichment and culture protocols, all of them utilized polyclonal Treg subsets that exhibit a plethora of different TCR specificities. The potential therapeutic benefit of polyclonal Treg population infusion relies on bystander immunosuppression which allows regulation by activated Treg cells through antigen-independent processes (47). Since polyclonal Treg cells undergo extensive activation and expansion *in vitro* prior to adoptive transfer, it is possible that they are capable of implementing this bystander effect. Therefore, a number of ongoing clinical studies are using polyclonal Treg cells for the treatment of AID including T1D (NCT02772679, NCT03444064), ulcerative colitis (NCT04691232) and Pemphigus (NCT03239470). However, growing evidence from animal models indicates that antigen-specific Treg cells may be more efficient in controlling pathological immune responses in a disease-specific manner (**Table 1**) (48–64). This is likely due to the migration of infused Treg cells towards tissues of cognate antigen exposure (49, 63) leading to more potent and localized control of inflammation with reduced risks of broad immunosuppression and associated adverse events. Moreover, the enhanced trafficking of antigen-specific Treg cells to target tissues presumably allows the administration of lower Treg cell numbers than polyclonal approaches, potentially facilitating the obtention of these cell numbers in standard *in vitro* expansion protocols. Nevertheless, the purification and expansion of disease-relevant antigen-specific Treg cells remains technically challenging because of their very low frequency in the peripheral blood (65). Therefore, current efforts are focusing on the generation of antigen-specific Treg cells *in vitro* by transformation of antigen-specific effector T (Teff) cells into cells with suppressive capacity (66–68), or genetic insertion of synthetic antigen receptors with disease-relevant antigenic specificities into isolated Treg populations (53, 64, 69) (**Figure 1D**).

GENERATION OF ANTIGEN-SPECIFIC TREG CELLS BY ANTIGEN-SPECIFIC EFFECTOR T CELL ENGINEERING

Similar to the development of pTreg cells *in vivo* several studies have demonstrated that both murine and human Treg cells can be generated from naïve CD4$^+$ T cells *in vitro* when they are stimulated in the presence of TGF-ß and IL-2 (induced Treg, iTreg) (66, 70). Hence, isolated antigen-specific effector T cells (Teff) could serve as a useful source to generate antigen-specific iTreg cells for adoptive cell therapy. However, it has become clear that the phenotype and function of iTreg cells is not properly maintained under inflammatory conditions (71–73). This is clinically relevant as iTreg cells might be able to regain their pro-inflammatory characteristics *in vivo* and contribute to an

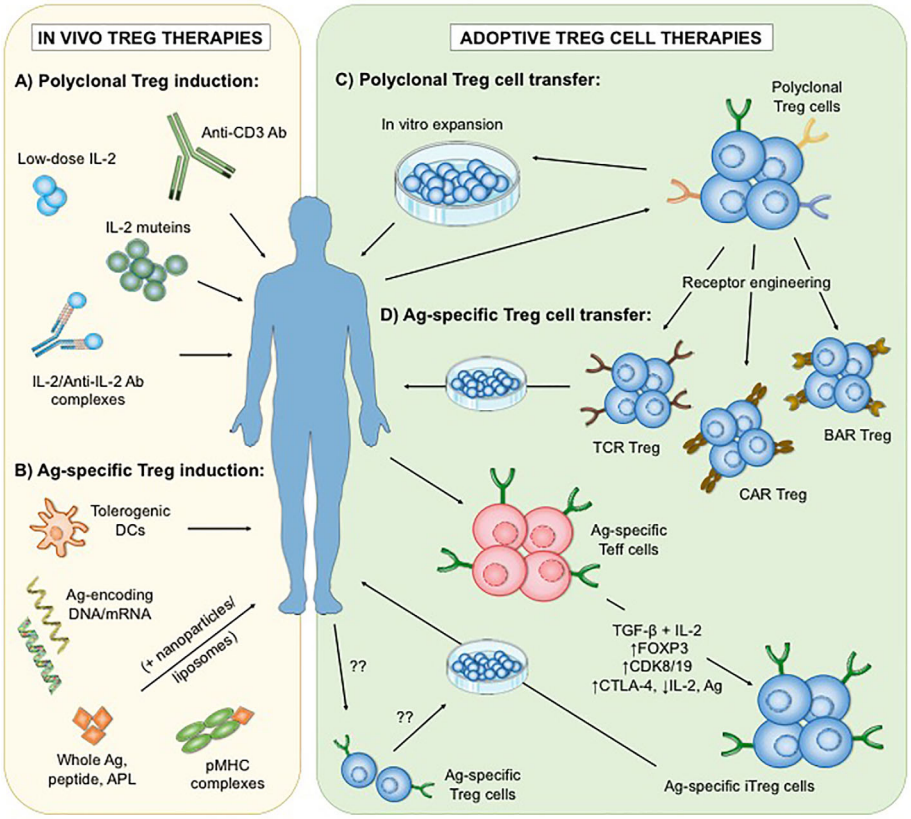

FIGURE 1 | Different approaches of polyclonal and antigen-specific Treg cell-based therapies. To date, two main strategies have been developed: the administration of immunomodulatory agents that enhance the number and/or function of Treg cells *in vivo* **(A, B)**, and the adoptive transfer of *in vitro* expanded Treg cells **(C, D)**. Interventions that increase polyclonal endogenous Treg cells *in vivo* involve low-dose interleukin-2 (IL-2), mutant IL-2, IL2/Anti-IL-2 Ab complexes as well as selective depletion of Teff cells by Anti-CD3 Ab **(A)**. In contrast, applications of antigen-based treatments could lead to the enhancement of antigen-specific Treg subsets **(B)**. On the other hand, adoptive Treg cell therapies rely on the optimal isolation and expansion of Treg cells *in vitro*. Thus far, clinical trials in autoimmunity have only utilized expanded polyclonal Treg cell populations **(C)**. However, antigen-specific Treg cells can be generated *in vitro* **(D)** by genetic insertion of synthetic receptors (including engineered T cell receptors (TCR), chimeric antigen receptors (CAR) or B cell antibody receptors (BAR)), or by transformation of antigen-specific effector T (Teff) cells into induced Treg (iTreg) cells *via* stimulation in the presence of transforming growth factor beta (TGF-ß) and IL-2, transgenic FOXP3 overexpression, blockade of cyclin-dependent kinase 8 (CDK8) and CDK19 signaling, or a combination of cytotoxic T lymphocyte antigen 4 (CTLA-4) overexpression, IL-2 ablation and antigenic stimulation. The isolation and expansion of endogenous antigen-specific Treg cells remains technically challenging. Ag, antigen; DCs, dendritic cells; APL, altered peptide ligands; pMHC, peptide-major histocompatibility complex.

augmented autoimmune response especially considering the inflammatory environment where they will be re-infused in. Thus, other strategies to re-program Teff lymphocytes into Treg cells have been developed including transgenic overexpression of FOXP3 *via* lentivirus-based techniques (74–78). While several studies demonstrated that FOXP3-transduced Teff cells exhibit Treg-like phenotypes and immunosuppressive functions, the random insertion of FOXP3 at different lentiviral integration sites might entail potential safety risks due to the heterogeneity of the final clinical product. Therefore, more advanced genetic tools, such as CRISPR/Cas9 or TALEN, have been recently utilized to generate FOXP3-expressing Teff cells *via* homology-directed repair-based gene editing (52, 79). Moreover, a CRISPR-based system has been shown to successfully repair the FOXP3 gene in T cells from IPEX (immune dysregulation polyendocrinopathy enteropathy and X-linked) syndrome patients (79). In addition, recent data have demonstrated the feasibility of generating human antigen-specific Treg cells from tetramer-enriched Teff populations by introduction of a transgenic FOXP3 promoter *via* TALEN and adeno-associated virus-based editing (52). It is noteworthy that Teff cells can also acquire Treg-like characteristics by FOXP3-independent approaches, including blockade of cyclin-dependent kinase 8 (CDK8) and CDK19 signaling pathways (67), as well as by a combination of CTLA-4 overexpression, IL-2 ablation and antigenic stimulation (80). All of these strategies were able to confer immunosuppressive functions to both naïve and activated Teff cells which retained their anti-inflammatory properties *in vivo* when transferred into different mouse models of autoimmunity (52, 67, 80). Nonetheless, it remains to be determined whether these applications would have clinical utility in human AID.

TABLE 1 | Pre-clinical studies demonstrating increased efficacy of antigen-specific adoptive Treg cell therapies for AID and transplantation.

Disease	Model	Antigen-specific Treg population	Evidence of superior function	Ref.
T1D	(BDC2.5) NOD mice	$CD4^+$ $CD25^+$ T cells from TCR-transgenic BDC2.5 mice expanded *in vitro* with BDC peptide and NOD DCs	Efficient inhibition of diabetogenic T cell-induced diabetes in NOD mice (no suppression with polyclonal $CD4^+$ $CD25^+$ NOD Treg cells)	(48)
T1D	(BDC2.5) NOD mice	$CD4^+$ $CD25^+$ T cells from TCR-transgenic BDC2.5 mice expanded *in vitro* with anti-CD3/CD28 beads	Enhanced suppression + reversal of diabetogenic T cell-induced diabetes in NOD.RAG-/- or NOD CD28-/- mice (only slight delay of disease with 4-fold higher numbers of polyclonal $CD4^+$ $CD25^+$ NOD Treg cells)	(49)
RA	DBA1 mice	$CD4^+$ T cells transduced with FOXP3 and a TCR of a CIA-associated T cell clone	Effective inhibition + reversal of CIA (no effect with FOXP3-transduced $CD4^+$ T cells without antigen specificity)	(50)
MS	(Tg4) B10.PL mice	$CD4^+$ $CD25^+$ T cells from TCR-transgenic Tg4 mice expanded *in vitro* with anti-CD3/CD28 beads	Potent inhibition + amelioration of MBP- or PLP-induced EAE (no effect with polyclonal B10.PL Treg cells)	(51)
MS	(2D2) C57Bl/6 mice	HDR-edited FOXP3-overexpressing T cells (edTreg) from TCR-transgenic 2D2 mice	Better suppression of Teff proliferation *in vivo* in MOG-induced EAE compared to polyclonal C57Bl/6 edTreg cells	(52)
MS	C57Bl/6 mice	MOG-specific CAR-engineered $CD4^+$ T cells with transgenic FOXP3 expression	Increased migration into the brain + better control of MOG-induced EAE than MOCK-treated $FOXP3^+$ T cells	(53)
Autoimmune Neuropathy	Lewis rats	$CD4^+$ $CD25^+$ T cells from rats expanded *in vitro* with PNM and IL-2	Amelioration of PNM-induced EAN (no effects with $CD4^+$ $CD25^+$ T cells expanded with irrelevant autoantigen)	(54)
Colitis	TNP-Tg BALB/c mice	CAR-engineered $CD4^+$ $CD25^+$ Treg cells specific for TNP	Protection from TNBS-induced colitis (no effect with control CAR Treg cells)	(55)
Colitis	CEABAC mice	CAR-engineered $CD4^+$ $CD25^+$ Treg cells specific for CEA	Enhanced colon homing + more efficient amelioration of Teff-mediated and AOM-DSS-induced colitis compared to control CAR Treg cells	(56)
AIG	(TxA23) BALB/c mice	TGF-ß-induced iTreg cells generated from $CD4^+$ T cells of TxA23 mice	Prevention of Teff cell-induced AIG (no suppression with polyclonal BALB/c iTreg cells)	(57)
Skin transplantation	BRG mice	CAR-engineered human $CD4^+$ $CD25^+$ Treg cells specific for HLA-A2	Reduced graft injury in a human skin xenograft model compared to polyclonal Treg cells	(58)
Skin transplantation	NRG mice	CAR-engineered human $CD4^+$ $CD25^+$ Treg cells specific for HLA-A2	Superior inhibition of allospecific immune responses than polyclonal Treg cells in human skin xenograft model	(59)
Skin transplantation/ GvHD	NSG mice	CAR-engineered human $CD8^+$ $CD45RC^{low}$ Treg cells specific for HLA-A2	More potent suppression of immune responses than control CAR Treg cells	(60)
GvHD	(OVA Tg) C57Bl/6 mice	TGF-ß-induced OVA-specific iTreg cells generated from $CD4^+$ T cells of OT-II mice	Better prevention of GvHD than polyclonal iTreg cells	(61)

T1D, type 1 diabetes; NOD, non-obese diabetic; RA, rheumatoid arthritis; MS, multiple sclerosis; AIG, autoimmune gastritis; TCR, T cell receptor; DCs, dendritic cells; CIA, collagen-induced arthritis; HDR, homology-directed repair; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; PLP, proteolipid protein; EAE, experimental autoimmune encephalomyelitis; CAR, chimeric antigen receptor; PNM, peripheral nerve myelin; EAN, experimental autoimmune neuritis; TNP, 2,4,6-trinitrophenol; TNBS, 2,4,6-trinitrobenzene sulphonic acid; CEA, carcinoembryonic antigen; AOM-DSS, azoxymethane-dextran sodium sulfate; TGF-ß, transforming growth factor beta; HLA, human leukocyte antigen; GvHD, graft versus host disease; OVA, ovalbumin.

GENERATION OF ANTIGEN-SPECIFIC TREG CELLS BY GENETIC ENGINEERING

A different approach to generate antigen-specific Treg cells *in vitro* involves the alteration of polyclonal Treg specificities by genetic introduction of synthetic receptors, including engineered TCRs and chimeric antigen receptors (CARs). For example, Treg cells transduced with an exogenous TCR isolated from human islet-specific $CD4^+$ T cell clones possess more potent antigen-specific suppressive capacities than polyclonal Treg populations (64). Furthermore, adoptive transfer of Treg cells engineered with a TCR specific for myelin basic protein can efficiently improve experimental autoimmune encephalitis (EAE) (81). Different reports from animal models of T1D and RA also demonstrate that TCR engineering can be successfully combined with the transduction of FOXP3 in order to convert Teff lymphocytes into immunosuppressive antigen-specific Treg-like cells (68, 82). Although these preclinical studies are encouraging, the translation of TCR-engineered Tregs into the clinic is somewhat limited by their major histocompatibility complex (MHC) restriction and the need to isolate and identify antigen-specific and disease-relevant TCRs.

On the other hand, the development of chimeric antigen receptors (CARs) enables the generation of engineered Treg cells that recognize their antigen directly (including whole proteins) in a non-MHC restricted manner (83). CARs consist of an extracellular single-chain variable antibody fragment (scFv) fused with an intracellular CD3 activation domain and (potentially multiple) co-stimulation domains. While it has been suggested that integration of the co-stimulatory molecule CD28 is essential for potent CAR Treg functions (84), the optimal design of CAR Treg cells is still under ongoing investigation (85). Nevertheless, based on their successful application and tolerable safety profiles in cancer treatments (86), the transduction of CARs may be considered a promising approach for future clinical administration of antigen-specific

Treg cells in AID and transplantation. Notably, whereas CARs possess a higher affinity for their cognate antigen than TCRs, data suggest that CARs require a greater density of antigen for their activation (87, 88) [reviewed in (89)]. Thus, the use of CAR Treg cells might be more beneficial for clinical settings where the relevant antigen is highly expressed in the target site while TCR-engineered Treg cells are potentially more efficacious in diseases associated with low antigen expression levels.

Initial studies in autoimmunity reported that CAR-engineered Treg cells specific for 2,4,6-trinitrophenol (TNP) can efficiently reduce murine colitis whereas this was not observed with irrelevant CAR Treg cells (55, 90). Similar results were obtained in two different experimental colitis models that utilized CAR Treg cells recognizing carcinoembryonic antigen (CEA) and confirmed the superior immunosuppressive function of CEA-specific Treg cells compared to non-specific control Treg cells. Moreover, histological analysis revealed that only CEA-CAR Treg cells were able to migrate to the inflamed colon of diseased animals (56). Furthermore, myelin oligodendrocyte glycoprotein (MOG)-specific CAR Treg cells have been shown to better control EAE than sham-treated Treg cells. In this study, CAR engineering was combined with the transgenic expression of FOXP3 in $CD4^+$ Teff cells resulting in MOG-specific immunosuppressive Treg cells that were able to home to the brain, to decrease EAE symptoms and to mediate protection from a second EAE challenge using pertussis toxin and complete Freund's adjuvant (53). In addition, HLA-A2 CAR-expressing ($CD4^+$ or $CD8^+$) Treg cells have been used in different pre-clinical studies of skin transplantation demonstrating superior suppression of human skin graft rejection and reduced GvHD in humanized mouse models (58–60). A phase 1/2a trial is currently examining the safety of HLA-A2 CAR-engineered autologous Treg cells in kidney transplantation (NCT04817774).

Strategies utilizing genetically engineered Treg cells with B cell antibody receptors (BARs) are also under ongoing investigation. Instead of the extracellular scFv used in CARs, BARs contain an antigen or antigen fragment that can be recognized by B cell receptors (BCRs) on inflammatory antibody producing B cells (91). Like CAR Treg cells, BAR Tregs are not MHC restricted and initial results demonstrated potent immunosuppression in mouse models of allergy (92) and hemophilia (69). Finally, although only currently studied in the context of T cells, and not Tregs, chimeric autoantibody receptor (CAAR) engineering could provide an additional approach to directly target autoreactive B cells in AID (93).

IN VIVO TREG CELL-BASED THERAPIES

While the development and conduction of adoptive Treg cell therapies are costly and laborious, immunomodulatory drugs that target key molecules of Treg maintenance have the potential to improve Treg-mediated immune tolerance *in vivo*. These treatments can increase the expansion and/or function of polyclonal or antigen-specific Treg subsets depending on the underlying mechanism of action targeted (**Figures 1A, B**, respectively). Due to the higher expression of CD25 (the alpha chain of IL-2 receptor) on Treg cells compared to Teff cell populations, interventions that promote Treg-specific IL-2 signaling constitute an attractive approach to improve the performance of the whole endogenous Treg cell pool. Nonetheless, because of its wide range of cellular targets including $CD4^+$ and $CD8^+$ effector T cells and natural killer cells, different strategies that avoid bystander activation of these pro-inflammatory subsets have been developed. These include the treatment with low-dose IL-2 (94–96), engineered IL-2 muteins (97–99) and IL-2/IL-2 antibody complexes (100, 101) that predominantly bind to CD25 over CD122 (IL-2R beta chain) and hence, preferentially induce the expansion of Treg population over Teff lymphocytes. Besides IL-2, some studies suggest that multiple other cytokines can promote the induction and/or suppressive function of antigen-specific Treg cells, including IL-4 (102), IL-5 (103), IL-7 (104), IL-12 (105), IL-15 (106) and IFN-γ (107). Furthermore, Treg cell homeostasis relies on several other signaling molecules that can be targeted to increase Treg cell performance *in vivo*, like mammalian target of rapamycin (mTOR) (108), phosphatase and tensin homolog (PTEN) and protein phosphatase 2A (PP2A) (109–111) as well as essential metabolites (e.g. kynurenine and adenosine) (112, 113). The activation of costimulatory [such as tumor necrosis factor receptor 2, TNFR2 (114)] or co-inhibitory receptors including T cell immunoreceptor with Ig and ITIM domains (TIGIT) (115) or programmed cell death 1 (PD-1) (116), predominantly expressed on the surface of Treg cells, also have the potential to promote the expansion and/or function of certain Treg subsets more selectively (117). In addition, interventions that preferentially inhibit pathogenic T cells over Treg cells could be beneficial for the amelioration of autoimmunity or graft rejection. For example, anti-CD3 antibody-mediated improvement of immune tolerance in animal models and patients with AID has been associated with the promotion of Treg cells, partially by selective Teff cell depletion (118, 119).

In contrast to these non-specific immunomodulatory therapies, multiple studies suggest that treatment with disease-associated antigens can lead to the induction of antigen-specific Treg cells without the risk of broad immunosuppression (120–123). Several promising strategies to administer different kinds of antigenic drugs have been shown to inhibit inflammation and disease in preclinical models, but antigen delivery in human studies did not result in the same level of clinical improvements to date, with some of them even leading to worsening of disease (124) (reviewed in (125)). However, some reports detected signs of therapeutic benefit and induced immunotolerance which was associated with the expansion of $FOXP3^+$ Treg cells (122, 123). In the phase 1/2 Pre-Point trial islet autoantibody-negative children that were genetically at risk to develop T1D received oral insulin for 3-18 months. Interestingly, insulin treatment led to an immune response without unwanted hypoglycemia and induced insulin- and proinsulin-responsive T cells that exhibited characteristics of Treg cells, including FOXP3 expression and

lack of CD127 and pro-inflammatory cytokines (122). Another study reported improved C-peptide retention and lower insulin use in new-onset T1D patients that were intradermally injected with an immunodominant proinsulin peptide compared to a placebo group (123). This clinical benefit was associated with increased FOXP3 expression in Treg cells and higher levels of IL-10 secretion following proinsulin stimulation. It is worth mentioning that antigen administration in animal models of T1D, EAE and collagen-induced arthritis have also resulted in the generation of immunosuppressive IL-10 producing Tr1-like cells (120, 126) which might as well be beneficial in human AID (127, 128).

CHALLENGES AND IMPROVEMENTS OF ANTIGEN-SPECIFIC TREG THERAPIES

In order to develop efficient antigen-specific Treg cell-based treatments, disease-associated autoantigens must be well identified and characterized. However, this has not been achieved for many AID, including MS and psoriasis. The choice of the most appropriate antigen is also limited by possible antigen spreading following initial tissue damage, which could hinder the success of therapies that are based on a single antigen. This hurdle could potentially be overcome by targeting multiple self-antigens at the same time (if applicable). Nevertheless, some studies suggest that the exact definition of disease-initiating antigens might not always be necessary as long as the administered intervention leads to the accumulation of activated Treg cells in the affected inflammatory tissues that can induce other immunoregulatory populations in a contact-independent manner. This 'infectious tolerance' has been observed in a murine model of colitis where TNP-specific CAR Treg cells were able to reduce 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis (55). Localized bystander suppression could be further supported by the transgenic introduction of appropriate surface molecules that are necessary for the migration of activated Treg cells into disease-specific inflamed sites. While critical signals of Treg cell trafficking to specific tissues remain insufficiently described, previous studies suggest that lymphocytes require the expression of the chemokine receptor CXCR3 in order to home to the brain of MS patients and the pancreatic islets of patients with T1D (129, 130). Thus, both efficacy and tolerability of Treg cell administration in these AID could be enhanced by engineering $CXCR3^+$ tissue-specific Treg cells. On the other hand, patients suffering from psoriasis might benefit from Treg cells expressing the homing receptors CCR4 and cutaneous lymphocyte antigen (CLA) which are necessary for migration into the skin (131, 132).

Importantly, uncertainties about the safety of Treg cell infusions and *in vivo* immunomodulatory interventions still remain and have to be investigated with caution. In particular, the *in vivo* maintenance and suppressive function of *in vitro* generated (polyclonal or antigen-specific) Treg populations is an essential factor for the toxicity and efficacy of adoptive cell therapies. While small molecules have been shown to enhance the stability of iTreg cells *in vitro*, gene editing tools could be utilized to generate Treg cell populations with better resistance to pathological Treg plasticity in inflammatory environments (133). Such potential strategies could include overexpression of FOXP3 as aforementioned, or the knockout of molecules involved in pro-inflammatory signaling pathways present in inflamed tissues of autoimmunity. Notably, although the underlying mechanisms of Treg deficiencies in many AID are not well understood, human studies have reported that cytokines like IL-12 and IL-6 can induce defective Treg functions *in vitro* (19, 134). Hence, ablation of receptors that bind these cytokines might avoid pathological Treg instability following adoptive transfer. Moreover, genetic engineering approaches could be utilized to integrate suicide gene cassettes that can be activated in the case of disease augmentation or severe adverse events caused by harmful immune suppression, such as cancer development or chronic infections (135).

On the other hand, combination therapies of Treg cell transfer with immunomodulatory drugs that reduce autoimmune inflammation or support Treg maintenance could reduce the risk of Treg instability *in vivo*. Recently, a report demonstrated that combinatory intervention with anti-CD3 antibody enabled improved engraftment of autoantigen-specific Treg cells in the islets of a mouse model of T1D (136). The potential of anti-CD3 combination has been further confirmed in the context of antigenic peptide-based therapies with increased expansion of $FOXP3^+$ insulin-specific Treg cells and more potent remission of murine autoimmune diabetes upon nasal administration of proinsulin combined with anti-CD3 treatment (137). In order to minimize the risks of severe side effects caused by immunomodulatory drugs, combination strategies that support the *in vivo* maintenance of transferred Treg cells more selectively can also be envisioned. For example, engineering of antigen-specific Treg cells with a mutant IL-2 receptor might enable specific potentiation of these infused cells in response to mutant IL-2 administration and thereby, avoid the activation of pro-inflammatory cells by wild type IL-2 (138).

A major obstacle for the development of successful antigen-specific Treg therapies is the substantial level of Treg cell heterogeneity demonstrated by the expression of different lineage-defining transcription factors, such as T-box expressed in T cells (T-bet) (139), GATA-3 (140) or retinoic acid receptor-related orphan receptor gamma (RORγt) (141), and varying levels of cell surface molecules, including co-inhibitory/co-stimulatory receptors such as PD-1 (142) and inducible T cell costimulator [(ICOS) (143)], as well as chemokine receptors including CXCR3 and L-selectin [(CD62L) (144–146)]. In addition, Treg cells can mediate their immunosuppressive effects *via* numerous mechanisms involving the secretion of anti-inflammatory cytokines (147–150), IDO (151) and granzymes (152, 153), the actions of the ectoenzymes such as CD39 and CD73 (154) and multiple inhibitory molecules, such as PD-1 (155) and CTLA-4 (156, 157). This suggests that at a given time point distinct subpopulations of $FOXP3^+$ Treg cells can be identified in an individual with specialized functions and maintenance requirements which might depend on their developmental origin, the type of immune response they are controlling (Th1, Th2, or Th17-mediated inflammation) (144),

or the tissue they reside in. Tissue-resident Treg cells have been found in multiple non-lymphoid tissues and organs of healthy individuals (e.g. the skin, gut, lungs, liver, adipose tissue and skeletal muscle) where they can control local inflammation, but also contribute to normal tissue homeostasis during non-inflammatory settings *via* mechanisms that are independent of their immunosuppressive functions (158–160). However, the critical maintenance factors and characteristics of tissue-resident Treg cells during health and autoimmunity are still largely unknown. Hence, it is uncertain whether antigen-specific iTreg cells or antigen-based treatments can induce tissue-specific mechanisms of Treg-mediated immune regulation and tissue homeostasis.

Moreover, the underlying causes of numerical and/or functional deficiencies of antigen-specific Treg cells in AID are not well understood and might differ between patients suffering from similar disease symptoms. This is a particularly important factor in the context of autologous adoptive Treg cell therapy as the administration of potentially defective Treg cells might not result in a desired therapeutic outcome. Hence, it is crucial to identify specific Treg defects in an individual and repair affected pathways during the *in vitro* generation/expansion of antigen-specific Treg cells before adoptive transfer. This personalized strategy could include genetic editing of molecules involved in Treg survival and fitness (such as pathways involved in IL-2 signaling and FOXP3 expression) as well as the insertion of potentially underexpressed chemokine receptors (e.g. CXCR3, CCR4, CLA) in order to increase their capacity to migrate into disease-relevant tissues.

CONCLUSIONS

In order to develop safe and efficacious antigen-specific Treg therapies, further in-depth studies of the biology of human Treg cells during physiological homeostasis and autoimmune pathogenesis are needed. This requires new strategies to characterize distinct Treg subsets, better approaches to identify disease-relevant antigens and Treg defects as well as optimized tools to investigate clinical outcomes. In particular, new Treg biomarkers and technologies which can monitor the migratory behavior and function of infused or endogenous Treg cells *in vivo* are necessary to identify potential pitfalls that might limit therapeutic benefits. Moreover, it is conceivable that patients with AID might require subject-specific Treg-based treatments that rely on the identification of the individual's underlying Treg deficiency. Nonetheless, the limitations of autologous Treg cell therapies could be circumvented by the use of allogeneic Treg populations with optimal MHC matching. In addition, the creation of universal Treg donor lines by genetic alterations of MHC molecules constitutes a possible strategy that deserves further investigation (discussed in (161)). Together with the ongoing efforts to develop technologies to optimally engineer human Tregs, future studies on the molecular and cellular mechanisms that control human Treg function, stability and maintenance will be critical to optimize current Treg cell-based treatments and to identify new Treg-specific targets amenable to therapeutic intervention.

AUTHOR CONTRIBUTIONS

CS and MD-V wrote the manuscript. All authors contributed to the article and approved the submitted version.

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NKG2D Enhances Double-Negative T Cell Regulation of B Cells

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Numerous studies reported a small subpopulation of TCRαβ$^+$CD4$^-$CD8$^-$ (double-negative) T cells that exert regulatory functions in the peripheral lymphocyte population. However, the origin of these double-negative T (DNT) cells is controversial. Some researchers reported that DNT cells originated from the thymus, and others argued that these cells are derived from peripheral immune induction. We report a possible mechanism for the induction of nonregulatory CD4$^+$ T cells to become regulatory double-negative T (iDNT) cells *in vitro*. We found that immature bone marrow dendritic cells (CD86$^+$MHC-II$^-$ DCs), rather than mature DCs (CD86$^+$MHC-II$^+$), induced high levels of iDNT cells. The addition of an anti-MHC-II antibody to the CD86$^+$MHC-II$^+$ DC group significantly increased induction. These iDNT cells promoted B cell apoptosis and inhibited B cell proliferation and plasma cell formation. A subgroup of iDNT cells expressed NKG2D. Compared to NKG2D$^-$ iDNT cells, NKG2D$^+$ iDNT cells released more granzyme B to enhance B cell regulation. This enhancement may function *via* NKG2D ligands expressed on B cells following lipopolysaccharide stimulation. These results demonstrate that MHC-II impedes induction, and iDNT cells may be MHC independent. NKG2D expression on iDNT cells enhanced the regulatory function of these cells. Our findings elucidate one possible mechanism of the induction of peripheral immune tolerance and provide a potential treatment for chronic allograft rejection in the future.

Keywords: CD4$^-$CD8$^-$ double-negative T cells, TCRαβ, MHC class II, NKG2D, dendritic cells, CD4$^+$ T cells, B cells

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INTRODUCTION

Approximately two decades ago, a novel subset of TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$ double-negative T (DNT) cells was identified in the peripheral lymphoid tissues of normal rodents and humans (1). These DNT cells lack the expression of CD4, CD8 and NK1.1 but express TCRαβ and CD3. Although these cells account for only 1% to 5% of the peripheral lymphocyte population, they play an important role in the induction of peripheral immune tolerance and participate in the regulation of inflammatory responses. For example, DNT cells significantly prolong allo- and xenogeneic graft survival, alleviate graft-versus-host disease, and prevent autoimmune diseases and cancer in an antigen-specific manner (2–8). Nonetheless, the origin of these DNT cells is controversial.

Some researchers reported that DNT cells originated from the thymus (9), and others argued that these cells are derived from peripheral immune induction (10).

Previous researchers reported that nonregulatory $CD4^+$ T cells might be induced to become DNT cells *in vitro* (11). These ex vivo-induced DNT cells (iDNT cells) exhibit a phenotype that is consistent with the physiological DNT cell phenotype and perform regulatory functions, including antigen-specific inhibition of T cell- and B cell-mediated immune responses (11–13). Nevertheless, the mechanisms of induction are not well understood.

Natural killer group 2-member D (NKG2D) is an activating receptor that is commonly expressed on all NK cells. NKG2D is also expressed on some subsets of NKT cells, activated murine $CD8^+$ T cells, activated human $CD8^+$ T cells, γδ T cells, murine macrophages, and a minor population of human $CD4^+$ T cells (14–19). NKG2D is a fundamental receptor that binds to a variety of stress ligands, including ULBP and human Rae1 in humans. Rae1, H60 and Mult1 are the ligands of NKG2D in mice (20). The binding of NKG2D to its ligands induces NK cells to secrete cytokines that promote killing activity. NKG2D on $CD8^+$ T cells acts as a costimulatory factor, and its binding leads to effector and memory T cell formation (21, 22). However, little is known about whether iDNT cells express NKG2D and bind to NKG2D ligands.

The present study examined the possible mechanisms of the induction of nonregulatory $CD4^+$ T cells to become iDNT cells *in vitro*. We also examined the regulatory effects of iDNT cells on B cells and the mechanism of iDNT cell regulation of B cells. Our results suggested that immature bone marrow dendritic cells ($CD86^+MHC\text{-}II^-$ DCs), rather than mature bone marrow DCs ($CD86^+MHC\text{-}II^+$), induce iDNT cells. MHC-II impedes induction, and iDNT cells may be MHC independent. We also found that a group of iDNT cells expresses NKG2D. These $NKG2D^+$ iDNT cells had a stronger ability to regulate B cells *via* NKG2D ligands than cells that did not express NKG2D.

MATERIALS AND METHODS

Mice

Male 6-week-old C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and maintained in specific pathogen-free animal facilities of Peking University People's Hospital. The Peking University People's Hospital Animal Ethics and Experimental Committee approved all animal experiments.

Reagents and Flow Cytometry

Lipopolysaccharide (LPS) was obtained from Sigma (USA, California). Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2 and IL-4 were obtained from PeproTech (USA). A quantitative polymerase chain reaction (q-PCR) kit was purchased from Invitrogen. To analyze single-cell suspensions of lymphocytes, antibodies against CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD127 (A7R34), TCRβ (H57-597), and CD25 (7D4) were used to distinguish T cells. Antibodies against CD19, IgM and IgD were used to sort naïve B cells. An anti-CD40 antibody was used to stimulate naïve B cells (BioLegend, USA). Anti-CD86 (GL7), anti-MHC-II (I-A/I-E), and anti-CD11c (N418) antibodies were used to sort mature bone marrow DCs. Anti-CD3 (17A2), anti-CD4 (GK1.5), anti-TCRβ (H57-597) and anti-NKG2D (CX5) antibodies were used to characterize iDNT cells. Anti-Rae1 (186107), anti-ULBP-1/MULT-1 (FAB2588R-100UG) antibodies were used to detect the protein expression of Rae1 and Mult1 on B cells.

Purification and Induction of Bone Marrow DCs

DCs isolated from mouse bone marrow were induced according to the methods of Lutz (23). Briefly, red blood cells were lysed, and bone marrow cells from male BALB/c mice were cultured with recombinant GM-CSF (10 ng/ml) and recombinant IL-4 (10 ng/ml) and treated with LPS (10 μg/ml) on day 6. Bone marrow DCs were harvested on day 7 *via* positive selection for CD11c, CD86 and/or MHC-II.

Preparation of iDNT Cells *In Vitro*

iDNT cells were prepared as previously described with minor modifications (11). Briefly, $CD4^+CD127^{hi}CD25^-$ T cells were obtained from the spleens of male C57BL/6 mice *via* flow cytometry sorting. The sorted T cells were cocultured with bone marrow DCs from BALB/c mice at a ratio of 1×10^5 T cells to 2.5×10^4 DCs for up to 7 days in 96-well round-bottom plates in complete RPMI 1640 (RPMI medium containing 10% FBS, 100 IU/ml penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine). Different concentrations of recombinant IL-2 (0, 50 ng/mL, 100 ng/mL or 200 ng/mL) were added to the mixed lymphocyte reaction (MLR) to assess the induction effect of IL-2. $TCR\alpha\beta^+CD3^+CD4^-CD8^-NKG2D^+$ and/or $TCR\alpha\beta^+CD3^+CD4^-CD8^-NKG2D^-$ iDNT cells were selected using flow cytometry sorting.

Evaluation of the Effects of iDNT Cells on Naïve B Cells *In Vitro*

To evaluate the effects of iDNT cells on B cells, freshly sorted $NKG2D^+$ or $NKG2D^-$iDNT cells were cocultured with different ratios of B cells for 18 hours with 5 ng/mL LPS in B cell medium (RPMI medium containing 10% FBS, 100 IU/ml penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 2 μg/mL anti-CD40 antibody). Apoptosis kits (Invitrogen, Catalog# V13241) were used to detect B cell apoptosis. The EdU Flow Cytometry Assay Kit (Invitrogen, Catalog# C10424) was used to analyze the proliferation of B cells in these mixed lymphocyte reactions. To observe the effects of $NKG2D^+$ or $NKG2D^-$ iDNT cells on naïve B cells and plasma cells, 10 ng/mL LPS was added to the B cell medium for 2 days. Anti-B220, anti-CD138, anti-CD86 and annexin V antibodies were used to analyze the mixed lymphocyte reaction.

q-PCR

Total RNA was extracted from freshly sorted $NKG2D^+$ iDNT cells or $NKG2D^-$ iDNT cells using TRIzol reagent (Invitrogen, Catalog# 15596018) according to a standard protocol. The extracted RNA

was reverse transcribed into cDNA, and q-PCR was performed to detect the expression of *Inf-γ*, *perforin*, *IL-17a*, *IL-2*, *IL-4*, *IL-10*, *IL-21* and *granzyme b*. The following primers were used for q-PCR: *Inf-γ* (forward: ATGAACGCTACACACTGCATC, reverse: CCATCCTTTTGCCAGTTCCTC), *perforin* (forward: AGCACAAGTTCGTGCCAGG, reverse: GCGTCTCTC ATTAGGGAGTTTTT), *IL-17a* (forward: TTTAACTCC CTTGGCGCAAAA, reverse: CTTTCCCTCCGCATTGACAC), *IL-2* (forward: TGAGCAGGATGGAGAATTACAGG, reverse: GTCCAAGTTCATCTTCTAGGCAC), *IL-4* (forward: GGTCTCAACCCCCAGCTAGT, reverse: GCCGATGATCTCT CTCAAGTGAT), *IL-10* (forward: GCTCTTACTGACT GGCATGAG, reverse: CGCAGCTCTAGGAGCATGTG), *IL-21* (forward: GGACCCTTGTCTGTCTGGTAG, reverse: TGTGGAGCTGATAGAAGTTCAGG), and *granzyme b* (forward: CCACTCTCGACCCTACATGG, reverse: GGCCCCC AAAGTGACATTTATT). RNA extracted from B cells stimulated with LPS (10 μg/mL) in B cell medium for 12 hours was also reverse transcribed to detect *H60* (forward: CTGAGCTATC TGGGGACCATAC, reverse: AGTCTTTCCATTCAC TGAGCAC), *Rae1* (forward: TTTGGGAGCACAA CCACAGAT, reverse: TAAAGTTGGCGGGCTGAAAGA),

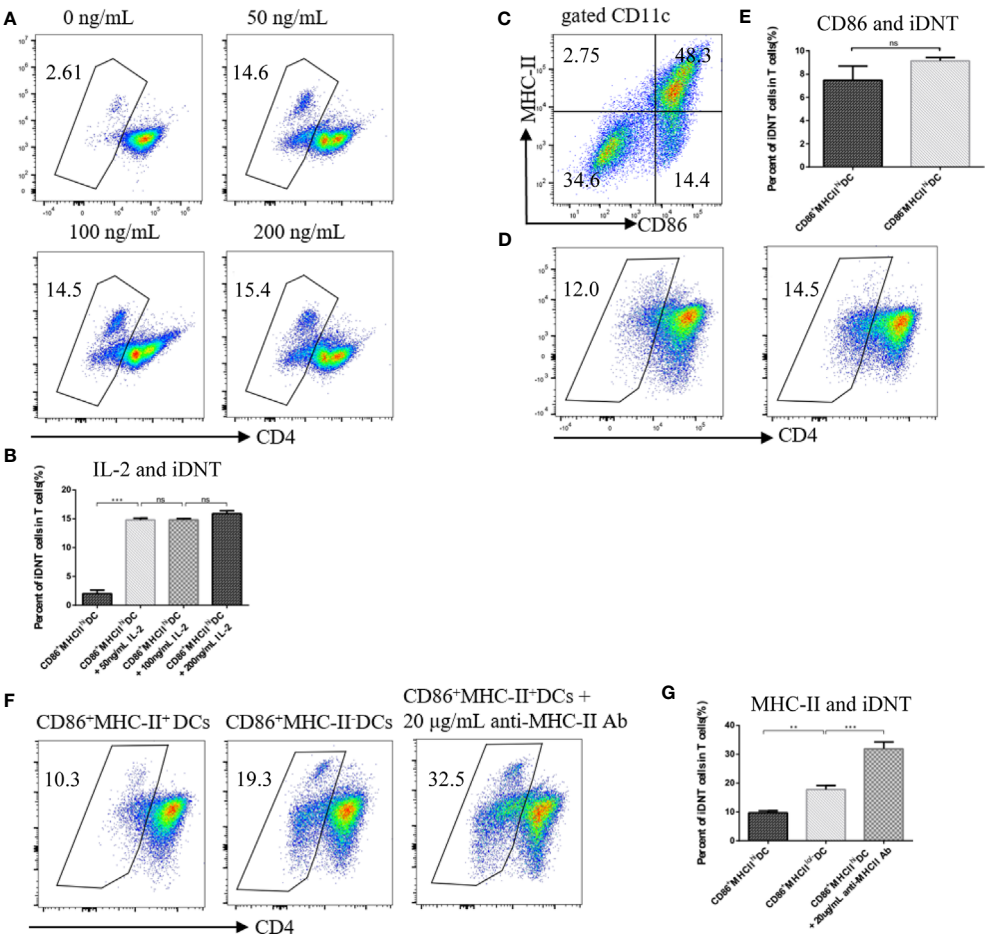

FIGURE 1 | MHC class II impedes the induction of nonregulatory $CD4^+$ T cells into iDNT cells. **(A, B)** Freshly sorted $CD4^+CD127^{hi}CD25^-$ T cells were cocultured with mature bone marrow dendritic cells ($CD86^-MHC\text{-}II^+$ DCs) at a ratio of 1×10^5 T cells to 2.5×10^4 DCs for up to 7 days in 96-well round-bottom plates in complete RPMI 1640. Different concentrations of IL-2 (0, 50 ng/mL, 100 ng/mL or 200 ng/mL) were added to the culture system. $TCR\alpha\beta^+CD3^+CD4^-CD8^-$ iDNT cells were analyzed to evaluate the influence of IL-2 on induction. **(A)** The $TCR\alpha\beta^+CD3^+CD4^-CD8^-$ cell population is gated. Representative flow cytometry profiles of different concentrations of IL-2 (0, 50 ng/mL, 100 ng/mL or 200 ng/mL). **(B)** The bar graphs are representative of three independent experiments. **(C)** Representative data of the use of anti-CD86 and anti-MHC-II antibodies to divide bone marrow DCs into two subgroups: mature DCs ($CD86^+MHC\text{-}II^+$) and immature DCs ($CD86^-MHC\text{-}II^+$, $CD86^+MHC\text{-}II^-$ or $CD86^-MHC\text{-}II^-$). **(D, E)** Immature DCs ($CD86^-MHC\text{-}II^+$) or mature DCs ($CD86^+MHC\text{-}II^+$) were cocultured with freshly sorted nonregulatory $CD4^+$ T cells. IL-2 (50 ng/mL) was added to the culture system. iDNT cells were analyzed to evaluate the influence of CD86 on induction. **(D)** Representative flow cytometry profiles of these two mixed lymphocyte reactions. **(E)** The bar graphs are representative of three independent experiments. **(F, G)** Immature DCs ($CD86^+MHC\text{-}II^-$), mature DCs ($CD86^+MHC\text{-}II^+$) or mature DCs with anti-MHC-II antibody (20 μg/mL) were cocultured with freshly sorted nonregulatory $CD4^+$ T cells. IL-2 (50 ng/mL) was added to the culture system. iDNT cells were analyzed to evaluate the influence of MHC class II on induction. **(F)** Representative flow cytometry profiles of these three mixed lymphocyte reactions. **(G)** The bar graphs are representative of three independent experiments. Student's *t*-test was used to compare two independent variables (ns, not significant, $^{**}p < 0.01$, and $^{***}p < 0.001$).

Mult1 (forward: CTGCCAGTAACAAGGTCCTTTC, reverse: GCTGTTCCTATGAGCACCAATG) and *GAPDH* (forward: AGGTCGGTGTGAACGGATTTG, reverse: TGTAGACC ATGTAGTTGAGGTCA) using q-PCR following the standard protocol provided by the manufacturer.

Statistical Analysis

All statistical analyses were performed using GraphPad v6.0 software. Data are presented as the means ± standard deviation. Student's *t*-test was used to compare two independent variables (ns, not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

RESULTS

MHC-II Impedes the Induction of $CD4^+CD127^{hi}CD25^-$ T Cells Into iDNT Cells

To examine the factors that influence the induction of nonregulatory $CD4^+$ T cells into $TCR\alpha\beta^+CD4^-CD8^-$ DNT cells, we added different concentrations of IL-2 to a culture system (**Figures 1A, B**). We found that the addition of 50 ng/mL, 100 ng/mL or 200 ng/mL IL-2 greatly affected the number of iDNT cells compared to no IL-2 treatment (**Figure 1B**). To further examine whether immature bone marrow DCs also induced this process, $CD86^-MHC\text{-}II^+$ DCs were cocultured with nonregulatory $CD4^+$ T cells (**Figure 1C**). We found no significant difference between $CD86^+MHC\text{-}II^+$ DCs and $CD86^-MHC\text{-}II^+$ DCs (**Figure 1D**). $CD86^+MHC\text{-}II^{lo/-}$ DCs were also used. We found that low or no MHC expression on $CD86^+$ DCs influenced the induction process (**Figures 1E, F**), and the addition of an anti-MHC-II antibody to the $CD86^+MHC\text{-}II^+$ DC group significantly increased the number of iDNT cells in the group compared to the numbers in other groups (**Figures 1E–G**).

IL-2 and MHC-II May Regulate NKG2D Expression on iDNT Cells

After the successful induction of iDNT cells, we found that some of these cells expressed NKG2D molecules (**Figure 2A**). Different concentrations of IL-2 and immature DCs revealed that IL-2 and MHC-II were related to NKG2D expression on iDNT cells

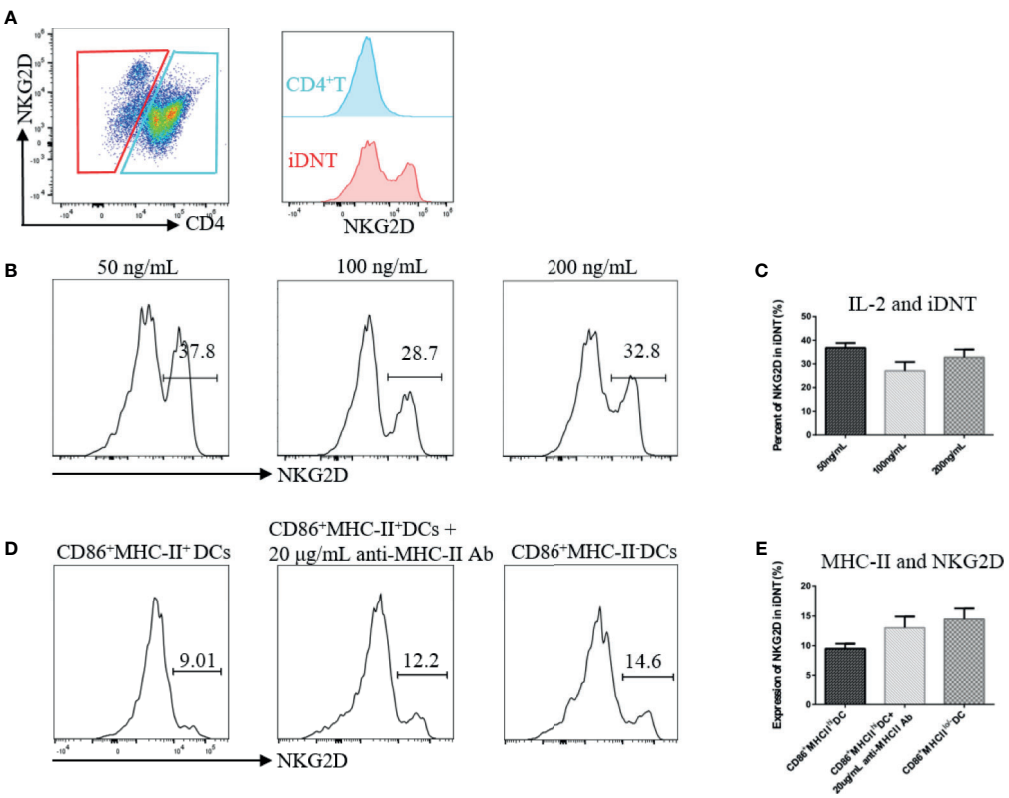

FIGURE 2 | IL-2 and MHC-II may regulate NKG2D expression on iDNT cells. **(A)** A representative experiment shows that a group of iDNT cells expressed NKG2D molecules. **(B, C)** Nonregulatory $CD4^+$ T cells were cocultured with mature bone marrow dendritic cells ($CD86^-MHC\text{-}II^+$ DCs). IL-2 (50 ng/mL, 100 ng/mL or 200 ng/mL) was added to the mixed lymphocyte reaction. After successful induction, the percentage of NKG2D on iDNT cells was detected using flow cytometry. **(B)** Histograms are representative of NKG2D expression on iDNT cells. **(C)** The bar graphs are representative of three independent experiments. **(D, E)** Immature DCs ($CD86^+MHC\text{-}II^-$), mature DCs ($CD86^+MHC\text{-}II^+$) or mature DCs with anti-MHC-II antibody (20 μg/mL) were cocultured with nonregulatory $CD4^+$ T cells. IL-2 (20 ng/mL) was added to the mixed lymphocyte reaction. After successful induction, the percentage of NKG2D on iDNT cells was analyzed using flow cytometry. **(D)** Histograms are representative of NKG2D expression on iDNT cells. **(E)** The bar graphs are representative of three independent experiments.

(**Figures 2B, E**). $CD86^+$ DCs with low or no MHC expression appeared to induce more iDNT cells than other types of DCs (**Figures 2B, E**).

NKG2D Enhances iDNT Cell-Mediated Inhibition of B Cell Proliferation and Promotes B Cell Apoptosis

To study the effects of NKG2D expression on iDNT cells on B cell proliferation and apoptosis, we sorted $NKG2D^+$ and $NKG2D^-$ iDNT cells and incubated these cells with naïve B cells at a ratio of 4×10^5 B cells to 1×10^5 iDNT cells in 96-well round-bottom plates in B cell medium (**Figures 3A, B**). EdU (10 μM) and LPS (5 μg/mL) were added to the medium, and B cell proliferation and apoptosis were detected approximately 18 hours later. We found that $NKG2D^+$ iDNT cells had a stronger ability to inhibit B cell proliferation and promote B cell apoptosis than $NKG2D^-$ iDNT cells. $NKG2D^-$ iDNT cells also suppressed B cell proliferation and promoted B cell apoptosis (**Figures 3C, D**).

NKG2D Enhances iDNT Cell-Mediated Inhibition of B Cell Differentiation Into Plasma Cells *In Vitro*

To further evaluate the function of NKG2D molecules, we extended the incubation time to 2 days, added a higher concentration of LPS (10 μg/mL) to the medium and reduced the number of B cells to a gradient ratio of 4×10^5 B cells to 0.4×10^5, 0.2×10^5 or 0.1×10^5 iDNT cells. We analyzed B cell differentiation and found that $NKG2D^+$ iDNT cells had a stronger inhibitory ability than $NKG2D^-$ iDNT cells at all ratios (**Figures 4A, B**). We also detected B cell apoptosis and found that $NKG2D^+$ iDNT cells promoted more B cell apoptosis than $NKG2D^-$ iDNT cells at all ratios. However, the difference in apoptosis between groups became less obvious as the ratio decreased (**Figures 4C, D**). We further evaluated CD86 to detect the level of activated B cells. As expected, iDNT cells suppressed B cell activation, and $NKG2D^+$ iDNT cells had a stronger inhibitory capability than $NKG2D^-$ iDNT cells (**Figure 4E**).

$NKG2D^+$ iDNT Cells Express Higher Levels of Granzyme B Than $NKG2D^-$ iDNT Cells, and Activated B Cells Express NKG2D Ligands

To clarify the mechanisms of $NKG2D^+$ DNT cell inhibition of B cell function, q-PCR was performed to examine granzyme B mRNA levels. We found that $NKG2D^+$ iDNT cells and $NKG2D^-$ iDNT cells expressed granzyme B, but $NKG2D^+$ iDNT cells expressed higher levels of granzyme B than $NKG2D^-$ iDNT cells (**Figures 5A, B**). This result is consistent with previous studies that showed that iDNT cells also expressed perforin. To determine whether activated B cells expressed NKG2D ligands, we used q-PCR to detect H60, Rae1 and Mult1. We found that activated B cells exhibited Rae1 expression (**Figures 5C, D**).

DISCUSSION

During antigen-presenting cell (APC) education, $CD4^+$ T cells are activated, proliferate, differentiate and secrete cytokines.

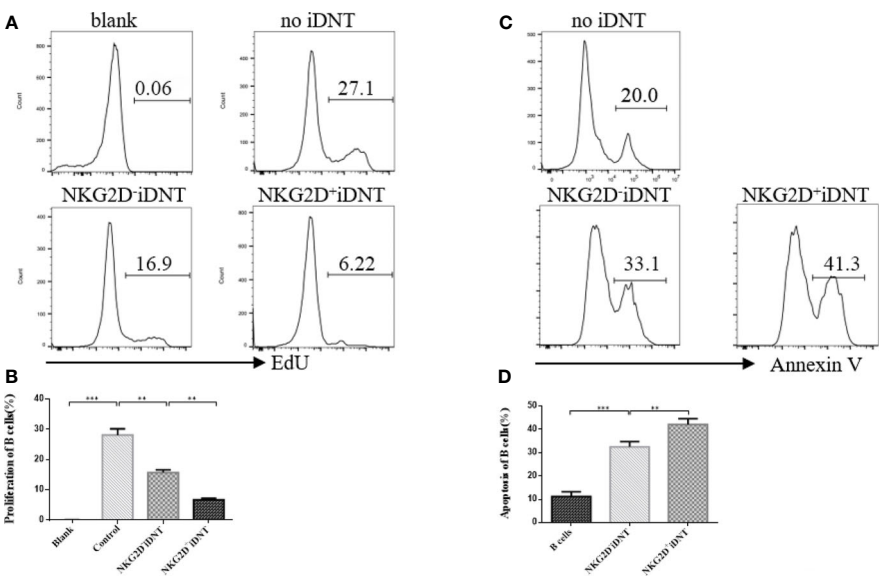

FIGURE 3 | NKG2D enhances iDNT cell-mediated inhibition of B cell proliferation and promotes B cell apoptosis. Naïve B cells were cultured with sorted $TCR\alpha\beta^+CD3^+CD4^-CD8^-NKG2D^+$ or $TCR\alpha\beta^+CD3^+CD4^-CD8^-NKG2D^-$ iDNT cells at a ratio of 4×10^5 B cells to 1×10^5 iDNT cells in 96-well round-bottom plates in B cell medium. EdU (10 μM) and LPS (5 μg/mL) were added to the medium, and B cell proliferation and apoptosis were detected approximately 18 hours later. **(A)** Histograms are representative of B cell proliferation. **(B)** The bar graphs are representative of three independent B-cell proliferation experiments. **(C)** Histograms are representative of B cell apoptosis. **(D)** The bar graphs are representative of three independent B-cell apoptosis experiments. Student's *t*-test was used to compare two independent variables (**$p < 0.01$ and ***$p < 0.001$).

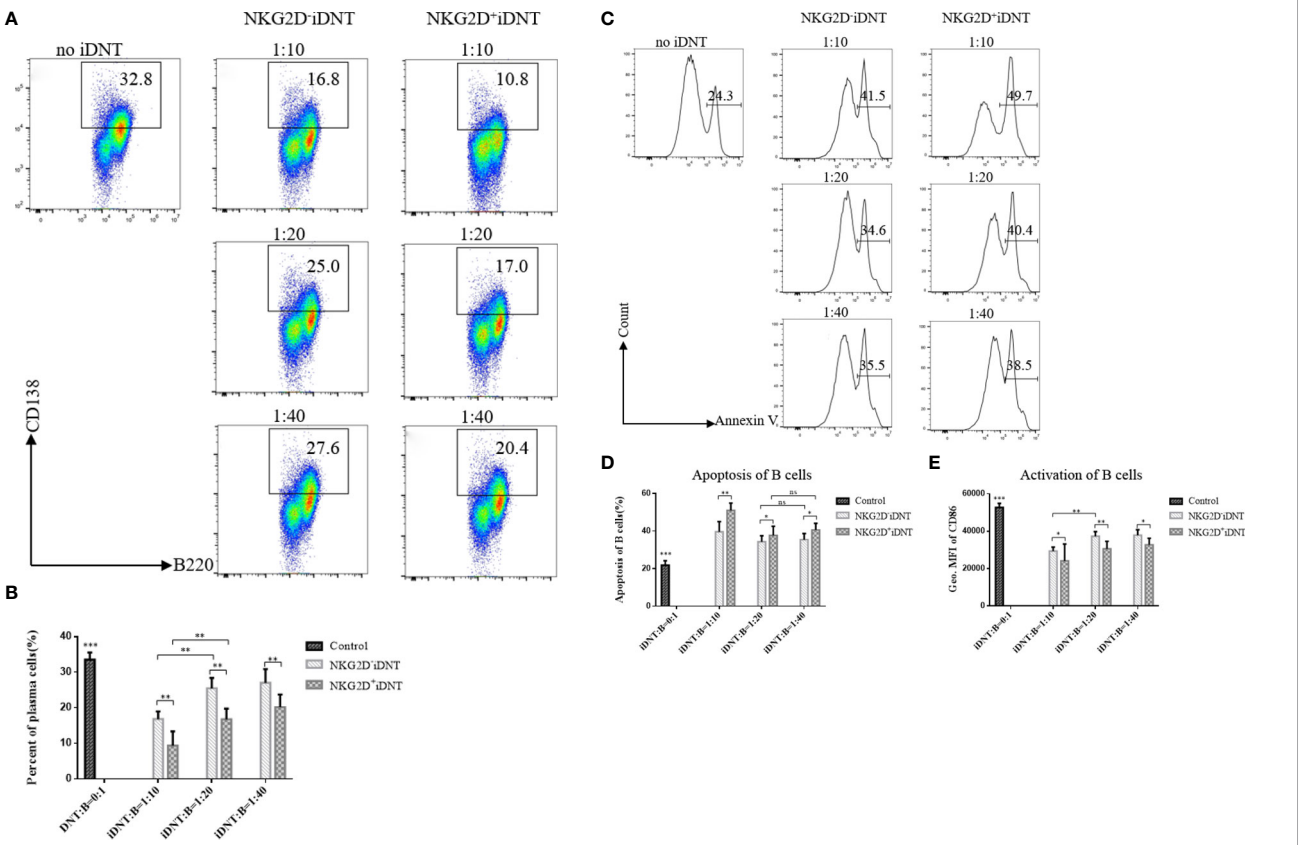

FIGURE 4 | NKG2D enhances iDNT cell-mediated inhibition of B cell differentiation into plasma cells *in vitro*. Freshly sorted TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$NKG2D$^+$ or TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$NKG2D$^-$ iDNT cells were incubated with naïve B cells at a gradient ratio of 0.4×10^5, 0.2×10^5 or 0.1×10^5 iDNT cells to 4×10^5 B cells in 96-well round-bottom plates in B cell medium for 2 days. LPS (10 μg/mL) was added to the medium, and we analyzed the activation, differentiation and apoptosis of B cells. **(A)** Representative flow cytometry profiles of B cell differentiation into plasma cells *in vitro*. **(B)** The bar graphs are representative of three independent B-cell differentiation experiments. **(C)** Histograms are representative of B cell apoptosis at different ratios. **(D)** The bar graphs are representative of three independent B-cell apoptosis experiments. **(E)** The bar graphs are representative of three independent B-cell activation experiments. Student's *t*-test was used to compare two independent variables (ns, not significant, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

All of these immune functions depend on at least two interactive signals between two types of cells: the first signal is derived from TCR-CD3 complexes, which are expressed on the surface of CD4$^+$ T cells and combine with peptide-MHC-II on APCs when activated; the second signal is transduced *via* CD86/80 molecules, which interact with CD28 molecules. Although several types of cells express MHC-II molecules, DCs are one of the most important APCs expressing this molecule and participating in CD4+ T cell immune responses. Once CD4$^+$ T cells recognize a foreign peptide-MHC-II complex on the plasma membrane of DCs, these cells form TCR-CD3-peptide-MHC-II complexes. CD4 molecules expressed on CD4$^+$ T cells restructure themselves and extend synapses with MHC-II molecules. Our study found that MHC-II$^{lo/-}$CD86$^+$ DCs, but not MHC-II$^+$CD86$^+$ DCs, enhanced induction. The addition of an anti-MHC-II neutralizing antibody to the CD86$^+$MHC-II$^+$ DC group significantly increased the number of iDNT cells (**Figures 1E, F**). These results suggested that CD4 molecules expressed on CD4$^+$ T cells "disappeared" because of low or nonexistent MHC-II expression. Unlike CD4$^+$ T cells binding to liver cell MHC-II molecules to form TCR-CD3-peptide-MHC-II complexes, which are later removed from liver cells in autoimmune hepatitis (24), MHC-II$^{lo/-}$CD86$^+$ DCs cannot efficiently contact CD4$^+$ T cells. Therefore, CD4 molecules cannot extend synapses with MHC-II molecules and may be gradually lost during the process of cell proliferation supported by a second signal and IL-2. Unlike many receptors that are internalized after continuous stimulation, the CD4 molecule present on the surface of CD4$^+$ T cells do not disappear *via* internalization (11). A recent study reported that concomitant disruption of the CD4 and CD8 genes facilitated the development of DNT cells in the periphery (25). This study used transgenic mice expressing human HLA class II molecules, HLA-DR3 or HLA-DQ8, as test subjects. Because human HLA class II molecules present superantigens more efficiently than murine MHC class II molecules, murine MHC class I and II molecules did not efficiently contact the coreceptors CD4/CD8, which led to CD4 and CD8 gene inactivation. These authors found that this disruption enabled

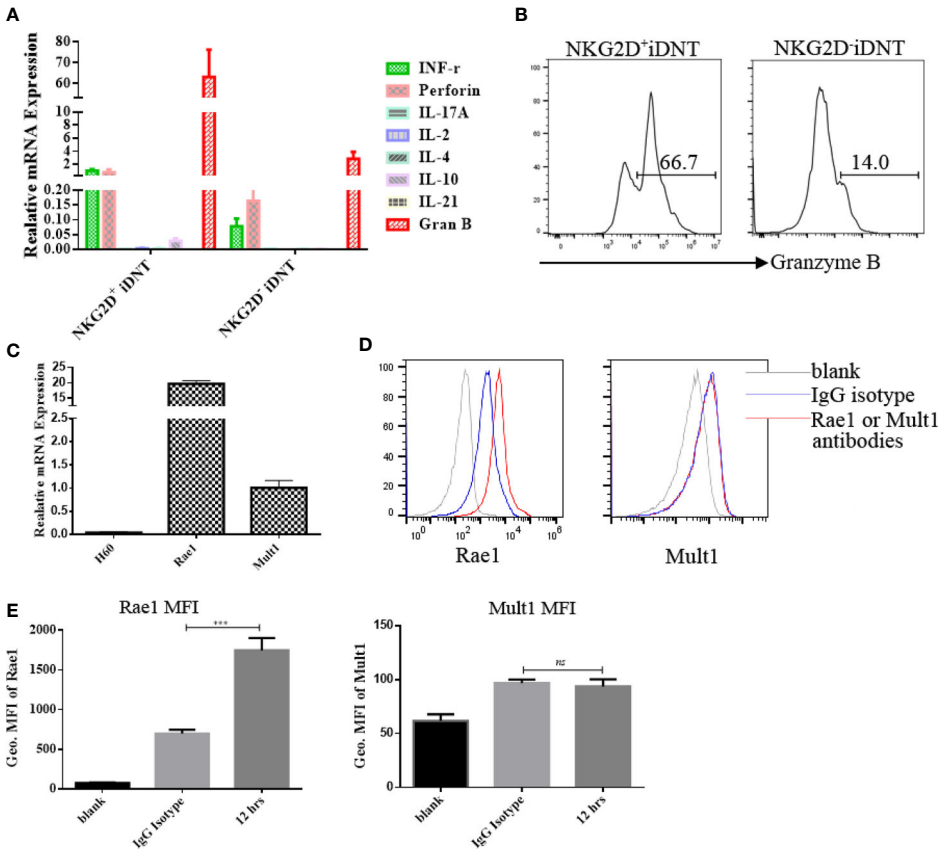

FIGURE 5 | NKG2D$^+$ iDNT cells express higher levels of granzyme B than NKG2D$^-$ iDNT cells, and activated B cells express the NKG2D ligand Rae1. **(A)** Q-PCR was performed to examine granzyme B mRNA levels in freshly sorted TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$NKG2D$^+$ and TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$NKG2D$^-$ iDNT cells. **(B)** Granzyme B protein was detected using flow cytometry. **(C)** Naïve B cells were stimulated with LPS in B cell medium for 12 hours, and q-PCR was performed to examine the expression levels of NKG2D ligands. **(D)** Naïve B cells were stimulated with LPS in B cell medium for 12 hours, and flow cytometry was performed to examine the expression of Rae1 and Mult1. **(E)** Geometric MFI of Rae1 and Mult1 protein. Student's t-test was used to compare two independent variables (ns, not significant, ***$p < 0.001$).

the development of DNT cells in the periphery, which is consistent with our findings.

Numerous studies reported that IL-2 was an important factor that promoted robust proliferation of CD4$^+$ T cells after interactions with DCs (26–28). The proliferative ability of treated cells increased gradually with increasing IL-2 concentrations. We also added IL-2 during the process of induction based on our previous studies (11, 12), but we found that different amounts of IL-2 did not obviously change induction (**Figures 1A, B**). Although DCs secrete IL-2 (27, 29, 30), this secretion is not sufficient. These results indicate that induction requires exogenous IL-2. Critically, anti-CD3 antibodies could not be added to the culture system when inducing CD4 molecule downregulation. This observation is very important because no iDNT cells were observed when anti-CD3 antibodies were added to the mixed lymphocyte reaction (unpublished data).

NKG2D is an activating receptor that is commonly expressed on NK cells. NKG2D is also present on NKT cells, activated CD8$^+$ T cells, γδ T cells, macrophages, and a small subgroup of CD4$^+$ T cells (20, 31, 32). However, little is known about whether TCRαβ$^+$CD3$^+$CD4$^-$CD8- T cells express NKG2D. Our study found that a group of iDNT cells expressed NKG2D molecules (**Figure 2A**), and the addition of IL-2 increased induction and NKG2D expression (**Figures 2D, E**). These results suggested that our iDNT cells were activated after successful induced. This result is similar to the results achieved with IL-2 administration, which activated CD8$^+$ T cells and increased the expression of NKG2D. We do not understand why MHC-II influenced the expression of NKG2D on iDNT cells (**Figures 2B, C**). However, we will study the mechanism of MHC-II influence on NKG2D in future work.

Activation of the NKG2D receptor promoted NK cell killing activity. This receptor enhanced effector and memory CD8$^+$ T cell formation (21, 22). Our study found a group of iDNT cells, NKG2D$^+$ iDNT cells, that promoted more B cell apoptosis and a stronger inhibition of B cell proliferation and plasma cell formation than NKG2D$^-$ iDNT cells (**Figures 3**, **4**). These results demonstrated that NKG2D enhanced iDNT cell-mediated regulation of B cells. We also determined why NKG2D$^+$ iDNT cells had a stronger regulatory function than NKG2D$^-$ iDNT cells. In this study, we found naïve B cells

upregulated the protein expression of the NKG2D ligand upon LPS stimulation (**Figures 5D, E** and **Supplementary Figures 1A–C**), which is consistent with a previous study that B cells were significantly stained with the NKG2D tetramer after stimulation of splenocytes with ConA or LPS (33). Our study also found that the transcript of Mult1 but not posttranslational protein was detected (**Figures 5C–E**), indicating the existence of translational or posttranslational regulation. A previous study reported that Mult1 protein was ubiquitinated and degraded under normal conditions (34). However, the degradation and ubiquitination was reduced in response to cell stress (34). In our research, stimulation of naïve B cells with LPS for 12 hours might not be sufficient to reduce the degradation and ubiquitination of Mult1 (**Supplementary Figures 1D, E**). $NKG2D^+$ iDNT cells and $NKG2D^-$ iDNT cells expressed granzyme B, and $NKG2D^+$ iDNT cells produced more granzyme B than $NKG2D^+$ iDNT cells (**Figures 5A, B**). These results suggest that $NKG2D^-$ iDNT cells are previously activated and secrete some granzyme B. NKG2D activation enhances $NKG2D^+$ iDNT cell regulation *via* NKG2D ligands on B cells by inducing an increased release of granzyme B. A recent study showed that the levels of NKG2D ligand expression on splenic B cells increased in mice with aging (35). Another study reported that a small subgroup of mouse B cells, B1a cells, which exhibit NKG2D and NKG2D deficiency, impaired B1a cell development and T cell-independent immune responses (36). All of these reports demonstrate that NKG2D regulates B cell development and effector B cells.

In summary, we showed that nonregulatory $CD4^+$ T cells may be induced to become regulatory iDNT cells *in vitro*. IL-2 promoted the induction process, and MHC-II expression on bone marrow DCs impeded this process. These iDNT cells were activated after successful induction, which promoted B cell apoptosis and inhibited B cell proliferation and plasma cell formation. A small portion of iDNT cells expressed NKG2D,

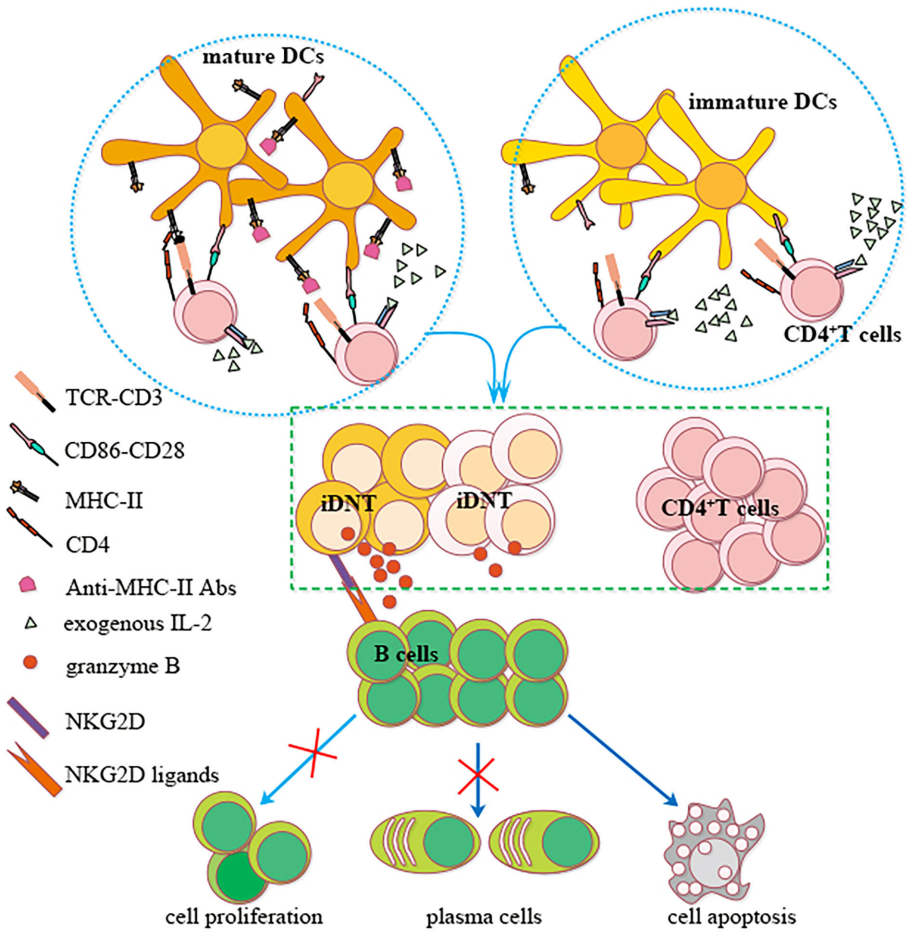

FIGURE 6 | A possible mechanism of nonregulatory $CD4^+$ T cells induction into iDNT cells and NKG2D enhancement of iDNT cells regulation of B cells. Nonregulatory $CD4^+$ T cells may be induced to become regulatory iDNT cells *in vitro*. IL-2 promotes the induction process, and MHC-II expressed on bone marrow DCs impedes this process. These iDNT cells were activated after successful induction, which promoted B cell apoptosis and inhibited B cell proliferation and plasma cell formation. A small portion of iDNT cells express NKG2D, which induces the release of granzyme B to enhance iDNT cell-mediated regulation of B cell functions *via* the NKG2D ligand Rae1.

which induced the release of granzyme B to enhance iDNT cell-mediated regulation of B cell functions *via* NKG2D ligands (**Figure 6**). Therefore, our research provides insight for understanding the mechanism of peripheral immune tolerance and the development of a potential treatment for chronic allograft rejection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Peking University People's Hospital Animal Ethics and Experimental Committee.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: S-hH and J-yZ. Performed the experiments: S-hH. Analyzed the data: L-hZ. Contributed reagents/materials/analysis tools: J-cG, JG, X-dX, QC, and XX. Wrote the article: S-hH and ZL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.650788/full#supplementary-material

Supplementary Figure 1 | The mRNA and Protein expression of Rae1 and Mult1. **(A–E)** Naïve B cells were stimulated with LPS (10 μg/mL) in B cell medium for 0, 6, 18 and 48 hours. Flow cytometry was performed to examine the expression of Rae1 and Mult1. **(A)** The protein expression of Rae1 was upregulated with the extension of culture time. **(B)** Geometric MFI of Rae1. **(C)** Percent of Rae1 protein expression with culture time. **(D)** The protein expression of Mult1. **(E)** Geometric MFI of Mult1. **Figures 1A–E** is an independent experiment. Student's t-test was used to compare two independent variables (ns, not significant, *p < 0.05, **p < 0.01, and ***p < 0.001).

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A Comparison of *Ex Vivo* Expanded Human Regulatory T Cells Using Allogeneic Stimulated B Cells or Monocyte-Derived Dendritic Cells

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Alloreactive regulatory T cells (arTregs) are more potent than polyclonal Tregs at suppressing immune responses to transplant antigens. Human arTregs can be expanded with allogeneic CD40L-stimulated B cells (sBcs) or stimulated-matured monocyte-derived dendritic cells (sDCs). Here, we compared the expansion efficiency and properties of arTregs stimulated *ex vivo* using these two types of antigen-presenting cells. Compared to sBcs, sDCs stimulated Tregs to expand two times more in number. The superior expansion-inducing capacity of sDCs correlated with their higher expression of CD80, CD86, and T cell-attracting chemokines. sBc- and sDC-arTregs expressed comparable levels of FOXP3, HELIOS, CD25, CD27, and CD62L, demethylated FOXP3 enhancer and *in vitro* suppressive function. sBc- and sDCs-arTregs had similar gene expression profiles that were distinct from primary Tregs. sBc- and sDC-arTregs exhibited similar low frequencies of IFN-γ, IL-4, and IL-17A-producing cells, and the cytokine-producing arTregs expressed high levels of FOXP3. Almost all sBc- and sDC-arTregs expressed CXCR3, which may enable them traffic to inflammatory sites. Thus, sDCs-arTregs that expand more readily, are phenotypically similar to sBc-arTregs, supporting sDCs as a viable alternative for arTreg production for clinical evaluation.

Keywords: immune regulation, regulatory T cell, Treg therapy, dendritic cells, B cells, human, transplantation, transplant tolerance

Abbreviations: APC, antigen-presenting cell; arTconv, alloreactive conventional $CD4^+$ T cell; arTreg, alloreactive regulatory T cell; CFSE, carboxyfluorescein diacetate, succinimidyl ester; cpm, counts per minute; EBV, Epstein Barr Virus; DCs, dendritic cells; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MFI, mean fluorescence intensity; MLR, mixed leukocyte reaction; MPLA, monophosphoryl lipid A; rh, human recombinant; sBcs, CD40L-stimulated B cells; sDCs, stimulated-matured monocyte-derived DCs; Tconv, conventional $CD4^+$ T cell; TCR, T cell receptor; Treg, regulatory T cell; TSDR, Treg-specific demethylated region.

INTRODUCTION

Organ transplantation can dramatically decrease morbidity and mortality, and improve the quality of life for patients with end-stage organ disease. However, in the process, the recipient's immune system is activated against donor alloantigens, leading to graft injury and potential graft loss (1). A combination of immunosuppressive drugs is currently used as standard therapy to prevent graft injury (2). However, the use of the current drugs to obtain optimum immune suppression is often limited by their toxicities. These drugs can enhance susceptibility to infection, injure organs *via* non-immune cell toxicities, and predispose individuals to development of cancer (2). For example, corticosteroids are toxic to pancreatic islets and can cause post-transplant diabetes (3). Calcineurin inhibitors exhibit nephrotoxicity and can consequently decrease the life of kidney grafts or impair renal function in recipients of other types of organ transplant (4). Also, corticosteroids and calcineurin inhibitors lead to frequent occurrence of metabolic (5) and neurologic (6) side effects, which have major impacts on the quality of life on the recipients of solid organ transplants.

Promoting immune tolerance to transplanted organs can potentially decrease or eliminate the use of immunosuppressive drugs. Several early phase regulatory T cell (Treg) therapy trials in transplantation have been initiated (7). In preclinical murine models, donor alloreactive-Tregs (arTregs) are 5-10 times more effective compared to polyclonal Tregs in reducing the number of anti-donor alloreactive T effector cells. In current clinical trials for solid organ transplantation, arTregs are being expanded using either irradiated donor PBMCs or donor-derived CD40L-stimulated B cells (sBcs) as antigen-presenting cells (APCs) (7). However, arTreg expansions can be highly variable (7), thus optimizing any aspect of the arTreg manufacturing process would be beneficial. Dendritic cells (DCs) are potent APCs that can expand arTregs (8–10). However, no study of our knowledge has directly compared Tregs activated by allogeneic B cells versus allogeneic DCs to determine what are the similarities and differences between these two approaches.

In this study, we compared the arTreg-stimulating capacity of human stimulated matured monocyte-derived DCs (sDCs) differentiated from $CD14^+$ blood monocytes, to that of sBcs to determine whether sDCs can potentially be used as an alternative APC to sBc for arTreg expansion.

MATERIALS AND METHODS

Cells

PBMCs from normal donors were isolated as previously described (11) under appropriate UCSF (Study 10-01980) and University of Pittsburgh (Study 19120084) institutional review board approval. Cells from males and females were used for all experiments, except only cells from females were used in the Treg-specific demethylated region (TSDR) methylation assay.

CD40L-Stimulated B Cells and Stimulated Matured Monocyte-Derived DCs

sBcs were generated as previously described using CD40L-expressing K562 cells (12). Cytokine-matured sDCs were used for all experiments, except monophosphoryl lipid A (MPLA)-matured sDCs were used in responder:stimulator combination 3 in the T cell receptor sequencing analysis. Cytokine-matured sDCs were generated from PBMC-isolated $CD14^+$ monocytes using the ImmunoCult™ Dendritic Cell Culture Kit (StemCell Technologies). Briefly, the kit contains a proprietary maturation supplement that includes TNFα and IL-1β. MPLA-matured sDCs were generated by differentiating monocytes in human recombinant (rh) IL-4- and rhGM-CSF-supplemented medium followed by maturation with MPLA. Prior to all assays, sBcs and sDCs were irradiated (25 Gray).

Mixed Leukocyte Reaction

Carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen/Thermo Fisher Scientific)-labeled responder PBMCs were cultured at 37°C with irradiated allogeneic sBcs (2 sBcs per PBMC) or sDCs (1 sDC per 4 PBMCs) for 4 days. CFSE dilution/proliferation was assessed by flow cytometry.

T Cell Culture

Tregs ($CD4^+CD127^{lo/-}CD25^+$) and conventional T cells (Tconvs; $CD4^+CD127^+CD25^-$) from responder PBMCs were FACS-purified using a FACS Aria II (BD Biosciences, San Jose, CA). The T cells were cultured with irradiated allogeneic sBcs (4 sBcs per 1 T cell) or sDCs (1 sDC per 4 T cells) in Optimizer T cell expansion media (Invitrogen), supplemented with rhIL-2 (300 IU/ml) (Proleukin, Novartis) at 37°C. Alloreactive T cell phenotypes were assessed on day 11.

sBc and sDC Cytokine Production

sBcs and sDCs were cultured alone at 37°C at the same density as used in T cell expansion cultures (200,000 sBcs or 12,500 sDCs in 100 μL assay medium). After 48 h, supernatants were harvested, and cytokine and chemokine levels were measured using 65-plex human cytokine/chemokine Luminex assay (Eve Technologies, Alberta, Canada).

Flow Cytometry

sBcs, sDCs, MLR cultures, and T cells were stained with antibodies against cell surface molecules, and, for some experiments, followed by intracellular stain for transcription factors and/or cytokines after fixation and permeabilization with Foxp3/Transcription Factor Staining Buffer Set (Invitrogen). Samples were analyzed on a BD Fortessa, BD LSRII or a Beckman Coulter Navios flow cytometer (Indianapolis, IN). Data analyses were performed using FlowJo (TreeStar, Ashland, OR) or Kaluza Analysis Software (Beckman Coulter). Precursor frequencies were calculated as previously described (13).

Treg-Specific Demethylated Region Methylation Assay

Frozen cell pellets were analyzed using the human FOXP3, Intron 1 TSDR region assay (EpigenDX, Hopkington, MA, ADS783-FS2) to obtain percentages of demethylated TSDR. All samples were from female donors. Due to X-chromosome inactivation in females, the maximum percentage of demethylation is ~50%.

Gene Expression Analysis of Stimulated T Cells

FACS-purified Tregs and Tconvs (primary-Tregs and primary-Tconvs) and expanded alloreactive T cells were stimulated with Dynabeads Human T-Activator CD3/CD28 beads (Invitrogen) for 24 h. RNA was isolated and analyzed using the Nanostring PanCancer Immune Profiling Panel (Seattle, WA). Nanostring data was analyzed using the nSolver 4.0 software.

Cytokine Analyses of Stimulated T Cells

For analysis of secreted cytokines, primary T cells and cultured alloreactive T cells were stimulated with anti-CD3/CD28 beads for 24 h. Supernatants were harvested and analyzed for cytokines and chemokines using a 42-plex Luminex assay (Eve Technologies). For analysis of intracellular cytokines, primary T cells and expanded alloreactive T cells were stimulated with PMA and ionomycin (Sigma Aldrich) in the presence of Brefeldin A (Sigma Aldrich) and monensin (BD Biosciences) for 5 h before staining and analysis using flow cytometry.

TCR Sequencing

RNA was isolated from ~$5x10^5$ arTregs using High Pure Isolation Kit (Roche Life Sciences, Indianapolis, IN) and submitted to iRepertoire (Huntsville, AL) for TCRβ sequencing and data analysis. Approximately 250,000 cell equivalent RNA was sequenced, which yielded ~$1x10^6$ reads after applying filters to eliminate sequencing artifacts per iRepertoire protocol. MiXCR software was used for TCR repertoire comparison and data visualization (14–16). Scripts developed in R were used to aggregate clones, plot data, and to calculate percentages of shared reads and unique CDR3s, and Jaccard and Morisita distances (17). Briefly, for each individual sample well, first, T cell clones with the same CDR3 amino acid sequence were merged. Second, the public clones (clone that is present in 2 or more samples) were extracted from the sample. Third, then the top 100 clones were extracted from the samples. Lastly, the filtered samples were compared with other samples. For digitally pooled samples, replicate well data were combined, then the filtering steps described for individual sample wells were performed.

In Vitro Suppression Assay

Responder PBMCs were cultured with irradiated stimulator PBMCs, in the presence of sBc- or sDC-arTregs for 7 days. ^{3}[H] thymidine (Perkin Elmer, Waltham, MA) was added for the final 16 h of culture. The arTregs tested were generated from the same donor as the responder PBMCs. sBcs or sDCs tested were generated from the same donor as the stimulator PBMCs. Additionally, third-party donors were used to assess specificity to the sBc and sDC donors. Proliferation was assessed using ^{3}[H] thymidine incorporation in triplicate wells and quantified as counts per minute (cpm). Percent suppression was calculated using the following formula = (1- [(mean cpm of wells with Tregs)/(mean cpm of wells with no Tregs)]) x100.

Statistics

Statistics were performed using GraphPad Prism, version 5 or 6 (GraphPad Software, San Diego, CA). Briefly, for most experiments, Wilcoxon matched-pairs signed rank test was used to compare sBcs versus sDCs, sBc-arTregs versus sDC-arTregs, and cytokine-producing cells versus non-cytokine-producing cells. In the Treg suppression assay, unpaired t-test was used to compare sBc-arTregs and sDC-arTregs at the same Treg dilution. Data from primary T cells and arTconvs are shown as a reference and were not included in statistical analyses.

RESULTS

Both sBcs and sDCs Are Potent Allogeneic T Cell Stimulators

We first compared the ability of sBcs and sDCs to stimulate allogeneic T cells by culturing mixed leukocyte reaction (MLR) cultures containing CFSE-labeled PBMCs with allogeneic sBcs or sDCs (**Figure 1A**). Previously, we found 2 sBcs per responding PBMC, and 4 sBcs per responding purified human Treg, were optimal ratios to stimulate T cell expansion (12). In contrast, one DC can stimulate 1-10 Tregs (9, 10). In pilot studies, we determined that 1:4, 1:8, and 1:16 ratios of sDC : PBMCs led to similar proliferation of T cells in PBMCs (**Supplementary Figure S1**). Thus, for all experiments described hereafter, for MLR cultures, we used ratios of 1 PBMC to 2 sBcs and 4 PBMCs to 1 sDC. For stimulating T cell expansions, we used ratios of 1 T cell to 4 sBcs, and 4 T cells to 1 sDC. We next compared the ability of sBcs and sDCs to drive cell cycle progression by back-calculating the frequencies of T cells that entered cycle in the original PBMC population based on division peaks (**Figure 1B**). sDCs, compared to sBcs, promoted more alloantigen-reactive $CD4^+$ T conventional cells (Tconvs), $CD8^+$ T cells, and in some cases $CD4^+FOXP3^+HELIOS^+$ Tregs to proliferate, but this was not statistically significant for Tregs. However, in most cases, responding Tconvs, $CD8^+$ T cells, and Tregs divided more when stimulated with sDCs, as indicated by a shift in the CFSE division peaks to the left (**Figures 1A, C**), and reflected in the increased proportions of divided T cells in sDC-stimulated cultures compared to sBc-stimulated cultures (**Figure 1D**). These data suggest that sDCs stimulate more T cells to enter the cell cycle and drive them to proliferate more in the 4-day culture. We next compared the two APCs' abilities to expand FACS-purified $CD4^+CD127^{lo/-}CD25^+$ Tregs and $CD4^+CD127^+CD25^-$ Tconvs. On average, sDCs, compared to sBcs, induced ~2-fold higher expansion of arTregs and arTconvs (**Figures 1E, F**).

We then compared sBcs and sDCs to explore what features of sDCs that might explain their higher T cell stimulatory capacities. Both sBcs and sDCs expressed comparable high levels of HLA-ABC and HLA-DR (**Figure 2A**). However, sDCs expressed higher levels of CD80 and CD86 and the adhesion molecule CD58. Robust T cell expansions are usually preceded by efficient clustering of T cells with APCs, which may be facilitated by chemokines (18). sBcs and sDCs secreted similar CCL5 levels on a per cell basis, whereas sDCs produced significantly higher levels of CCL3, CCL4, CCL17, and CCL22 (**Figure 2B**). Thus, higher expression of CD80, CD86 and CD58 by sDCs and their greater chemokine production may explain the higher potency of sDCs in stimulating arTreg expansion.

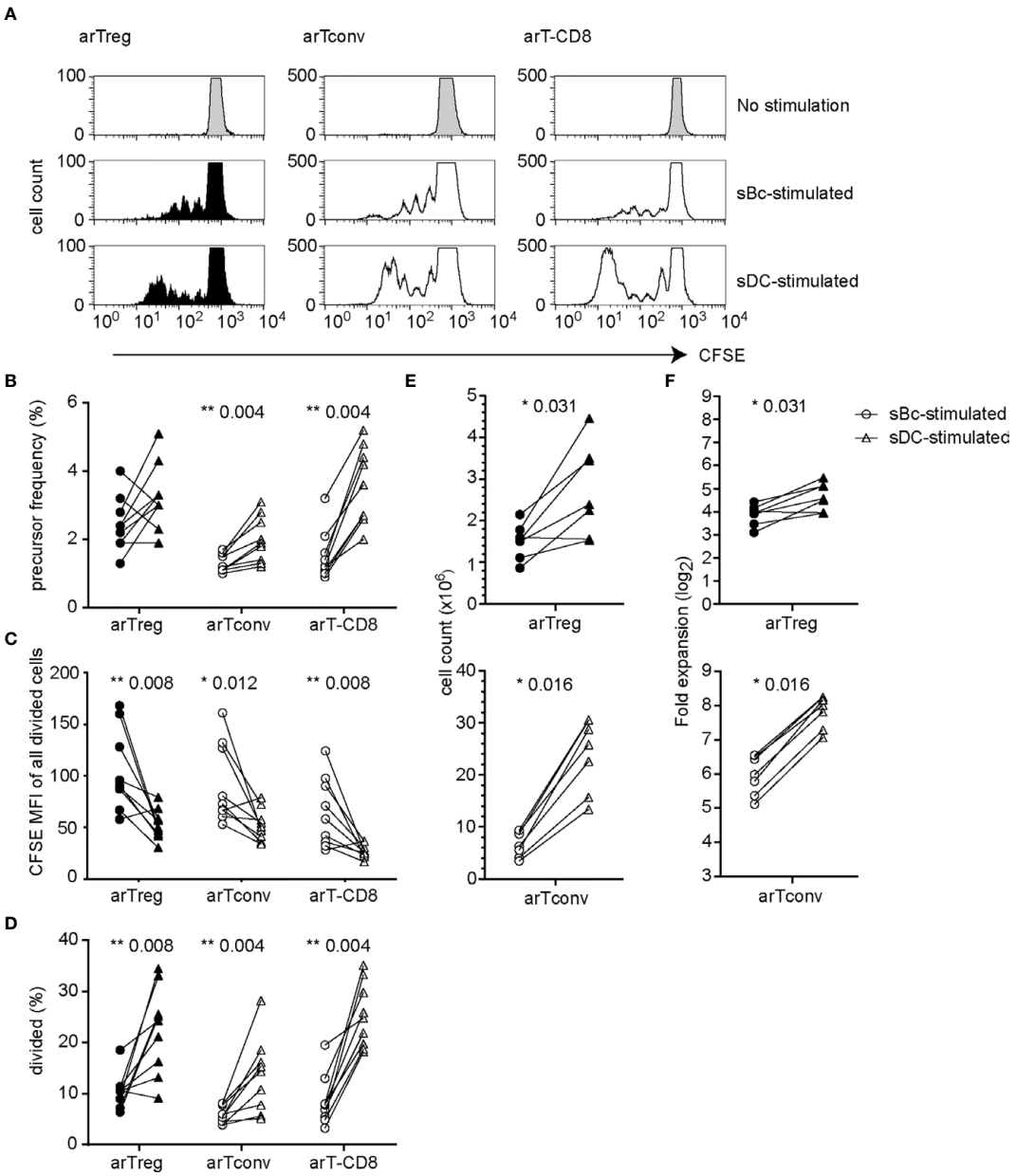


FIGURE 1 | sDCs, compared to sBcs, stimulate more alloreactive T cell proliferation. **(A–D)** CFSE-labeled responder PBMCs were stimulated with sBcs (2 sBcs per 1 PBMC), or sDCs (1 sDC per 4 PBMCs) for 4 days. **(A)** Representative histograms showing CFSE dilution/proliferation of T cells: Tregs ($CD3^+CD4^+CD8^-FOXP3^+HELIOS^+$), Tconvs ($CD3^+CD4^+CD8^-$non-Treg), and $CD8^+$ T cells ($CD3^+CD8^+CD4^-$). Histograms are zoomed in to show proliferation peaks. **(B)** T cell precursor frequency. **(C)** Divided T cells were gated as $CFSE^{lo}$, and CFSE level of expression was measured by mean fluorescence intensity (MFI). **(D)** Percentage of divided T cells ($CFSE^{lo}$) of total T cells. **(E, F)** FACS-purified Tregs ($CD4^+CD127^{lo/-}CD25^+$) and Tconvs ($CD4^+CD127^+CD25^-$) were cultured with sBcs (4 sBcs per 1 T cell), or sDCs (1 sDC per 4 T cells). arTreg (top) and arTconv (bottom) **(E)** cell counts on d11 and **(F)** fold expansion from d0 to d11. Cell counts were normalized to d0 count of 100,000. Data in **(A–D)** contain 9 different responder-stimulator combinations. Data in **(E, F)** contain 7 different responder-stimulator combinations. Connecting lines indicate alloreactive T cells stimulated by APCs (sBcs or sDCs) derived from the same donor. Statistics were performed using the Wilcoxon matched-pairs signed rank test.

arTreg Identity and Phenotype

Both sBc- and sDC-arTregs expressed high levels of the Treg lineage-defining transcription factor, FOXP3, and Treg-associated molecules HELIOS, CD25, CD27 and CD62L (**Figures 3A–D**). FOXP3 is also induced in arTconvs (19–21), but not expressed as highly as in arTregs (**Figures 3A–C**). A more definitive determination of Treg identity is the demethylation of the Treg-specific demethylated region (TSDR), an enhancer in

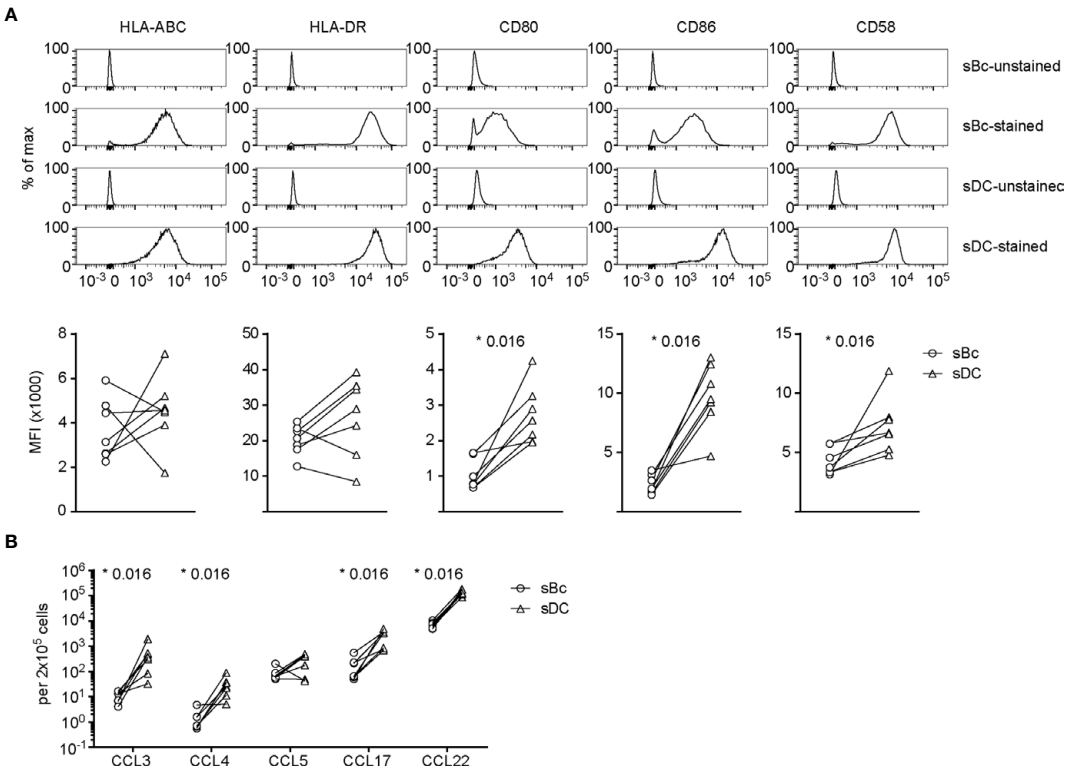


FIGURE 2 | sDCs, compared to sBcs, express higher levels of costimulatory molecules and secrete more T cell-attracting chemokines. **(A)** Expression of cell surface molecules on sBcs and sDCs. Cells were stained with antibodies ("sBc-stained" and "sDC-stained"), or were not stained with antibodies ("sBc-unstained" and "sDC-unstained") for reference. Representative histograms (top row) and level of expression (MFI) (bottom row) of cell surface molecules on sBcs and sDCs. **(B)** Secretion of chemokines by sBcs and sDCs. sBcs and sDCs were plated alone at the same density used to culture purified T cells (200,000 sBcs or 12,500 sDCs in 100μL assay medium). After 48 h, the culture supernatants were harvested and tested for the indicated molecules using Luminex assay. Data in **(A, B)** contain 7 different sBc:sDC pairs. Connecting lines indicate sBcs and sDCs derived from the same donor. Statistics were performed between sBcs and sDCs using the Wilcoxon matched-pairs signed rank test.

the FOXP3 gene. sBc- and sDC-arTregs displayed similar high percentages of demethylated TSDR (**Figure 3E**), suggesting that both sBc and sDC expanded bona fide lineage-committed arTregs.

To further probe the phenotype of the sBc- and sDC-expanded arTregs, we restimulated them with anti-CD3/CD28 beads for 24 h, then examined their gene expression using a 770-gene panel from Nanostring (**Table S1**). Unsupervised clustering analysis showed that sBc- and sDC-arTregs were most similar and distinct from arTconvs, and further separated from Tregs and Tconvs not expanded by APCs (primary Tregs and Tconvs) (**Figure 4A**). Consistent with protein expression assessed before restimulation (**Figures 3A–D**), mRNA expression of Treg-associated molecules FOXP3, CD25, CD27, and CD62L were mostly similarly expressed between sBc- and sDC-arTregs (**Figure 4B**). Additionally, sBc- and sDC-arTregs expressed mRNA encoding other Treg-associated molecules, such as GITR (glucocorticoid-induced tumor necrosis factor receptor, TNFRSF18), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), TIGIT (T Cell Immunoreceptor With Ig And ITIM Domains), and CD39 (**Figure 4B**).

Previous studies have shown that repeated *in vitro* stimulation of Tregs leads to Treg destabilization (22, 23). To assess potential arTreg destabilization after restimulation, we examined expression of molecules that are normally repressed in Tregs. Upon activation, Tconvs preferentially express CD40L compared to Tregs (24). sBc- and sDC-arTregs expressed lower levels of CD40L mRNA compared to sBc- and sDC-arTconvs (**Figure 4C**). Additionally, sBc- and sDC-arTregs expressed lower levels of IL-2 and IL-7R mRNA compared to sBc- and sDC-arTconvs, consistent with low IL-2 in arTreg culture supernatants (**Figure 4F**). Previous studies have shown that stable Tregs are characterized by lower expression of STAT4 protein compared to Tconvs (25). Interestingly, STAT4 mRNA induction was largely similar between arTregs compared to arTconvs (**Figure 4C**).

Although sBc- and sDC-arTregs expressed similar levels of Treg-associated molecules, we found 23 genes that were differentially expressed by at least 2-fold between sBc- and sDC-arTregs (**Figures 4D, E**). Notably, mRNA encoding CD38, a transmembrane cyclic ADP ribose hydrolase, was induced almost 7-fold more in sBc-arTregs compared to sDC-arTregs (**Figure 4D**). Previous studies have shown that mouse $CD38^+$ Tregs are more suppressive than $CD38^-$ Tregs (26), which suggest sBc-arTregs may be more suppressive than sDC-arTregs.

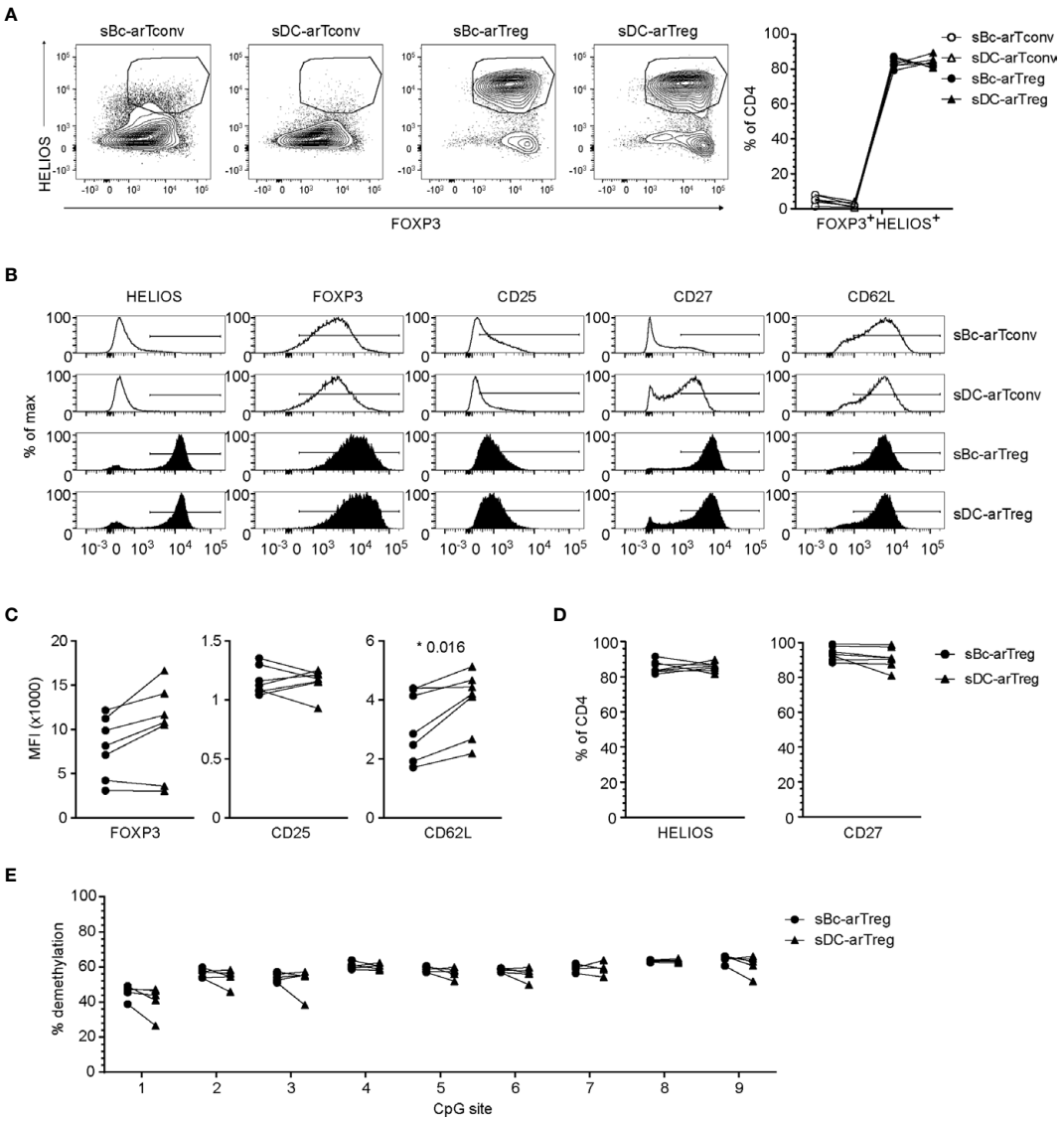
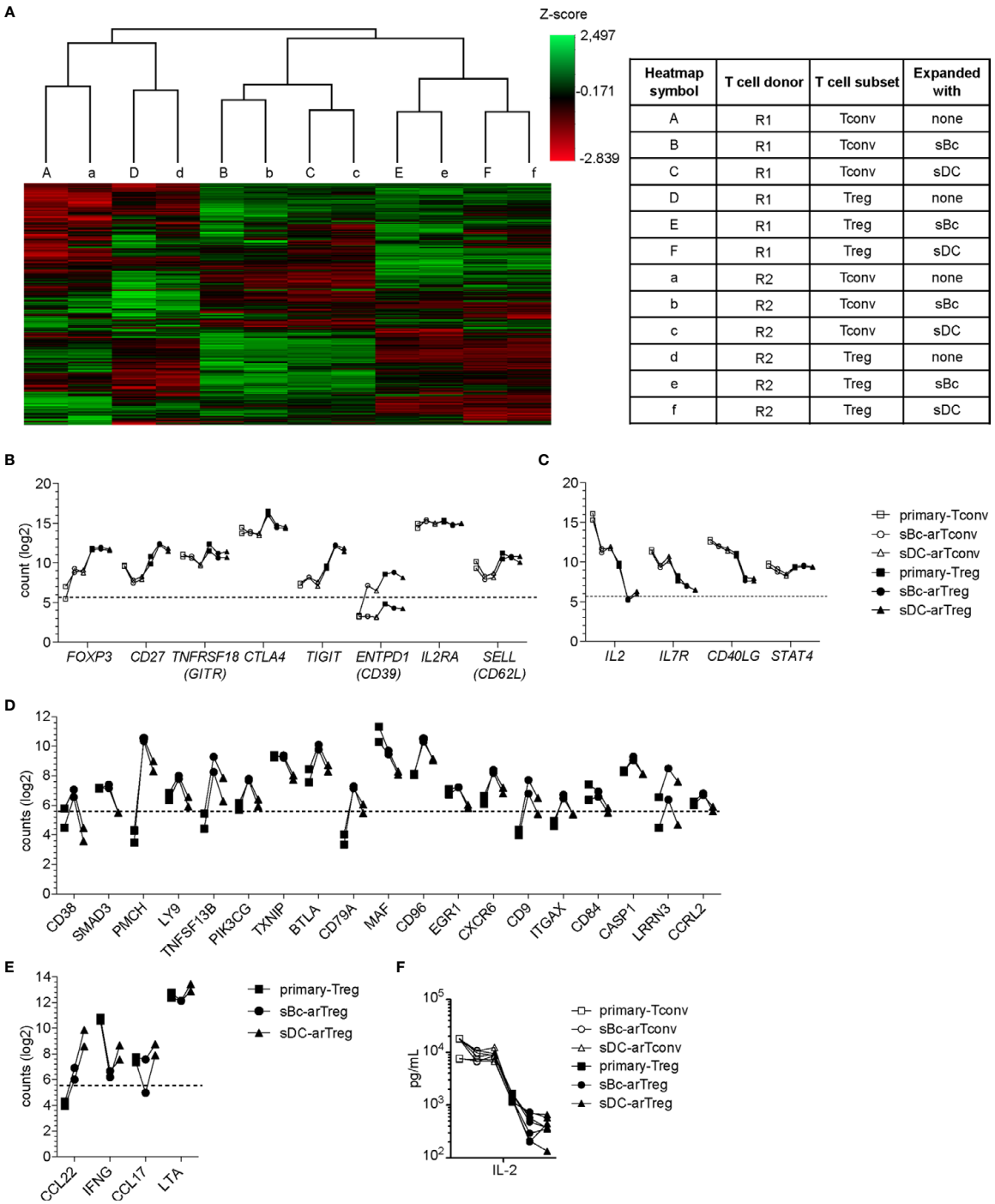


FIGURE 3 | sBc- and sDC-arTregs express Treg-associated molecules and are stable Tregs. **(A–D)** Expression of different molecules by arTregs and arTconvs was assessed by flow cytometry. **(A)** Representative FOXP3 versus HELIOS contour plots of arTregs and arTconvs (left) and %FOXP3$^+$HELIOS$^+$ of CD4 cells (right). **(B)** Representative histograms of arTregs and arTconvs. **(C)** Level of expression (MFI) and **(D)** Percentage of CD4$^+$ T cells expressing specific molecules. **(E)** Percentage of demethylated FOXP3 gene TSDR at different CpG sites. All arTregs were derived from female donors so maximum demethylation was ~50%. Data in **(A–D)** contain 7 different responder-stimulator combinations. Data in **(E)** contain 5 different responder-stimulator combinations. Connecting lines indicate alloreactive T cells stimulated by APCs (sBcs or sDCs) derived from the same donor. Statistics were performed between sBc-arTregs and sDC-arTregs using the Wilcoxon matched-pairs signed rank test. Data from arTconvs are shown as reference and were not included in statistical analyses.

Interestingly, we found sDC-arTregs compared to sBc-arTregs expressed almost 7-fold higher mRNA levels encoding a CCR4 ligand, CCL22 (**Figure 4E**). Higher expression of CCL22 may allow sDC-arTregs attract CCR4-expressing T cells (18) to the same APC by which they have been activated. All other chemokines in the Nanostring panel either showed no difference between sBc- and sDC-arTregs (CCL1, CCL3, CCL4, CCL5, CCL17, CCL20, CCL22), or were below background (data not shown).

arTreg Specialization

Tregs can specialize to suppress specific T effector cell functions (7). Specialized Tregs express transcription factors, cytokines and chemokine receptors that are associated with the CD4$^+$ T effector subsets they suppress (27). The tissue microenvironment where Tregs are activated influences Treg specialization, thus we examined the cytokine secretion profile of sBcs and sDCs. sDCs, compared to sBcs, expressed higher levels of IL-1β, IL-6, IL-12p70, and IL-18 (**Figure 5A**). sDC cultures also produced more IL-1R antagonist (IL-1RA).



Heatmap symbol	T cell donor	T cell subset	Expanded with
A	R1	Tconv	none
B	R1	Tconv	sBc
C	R1	Tconv	sDC
D	R1	Treg	none
E	R1	Treg	sBc
F	R1	Treg	sDC
a	R2	Tconv	none
b	R2	Tconv	sBc
c	R2	Tconv	sDC
d	R2	Treg	none
e	R2	Treg	sBc
f	R2	Treg	sDC

FIGURE 4 | sBc- and sDC-arTregs maintain their Treg identity after restimulation. **(A–F)** FACS-purified T cells (primary-Tregs and primary-Tconvs) and cultured alloreactive T cells were stimulated with anti-CD3/CD28 beads for 24 h. **(A–E)** RNA was harvested from stimulated cells and analyzed using Nanostring's PanCancer Immune Profiling Panel. **(A)** Heatmap generated from unsupervised clustering analysis of 439 normalized gene expression data (left). Description of T cell subsets and APC stimulation (table, right) **(B)** Gene expression of Treg-associated molecules. **(C)** Gene expression of Tconv-associated molecules. **(D)** Genes differentially expressed at least 2-fold greater in sBc-arTregs compared to sDC-arTregs. **(E)** Genes differentially expressed at least 2-fold less in sBc-arTregs compared to sDC-arTregs. **(F)** Supernatants from stimulated cells were collected and analyzed for IL-2 using Luminex assay. Data in **(A–F)** contain 2 different responder-stimulator combinations. Values above the dotted line are above background expression. Data in **(F)** contain 7 different responder-stimulator combinations. Connecting lines indicate alloreactive T cells stimulated by APCs (sBcs or sDCs) derived from the same donor. Statistics in **(F)** were performed between sBc-arTregs and sDC-arTregs using the Wilcoxon matched-pairs signed rank test. Data from primary T cells and arTconvs are shown as reference and were not included in statistical analyses.

We then compared sBc- and sDC-arTregs for their specialization phenotypes. We first examined the molecules associated with T_H1-like Tregs. Both sBc- and sDC-arTregs expressed less TBX21 when compared with primary Tregs and Tconvs, but sDC-arTregs expressed ~2-fold more TBX21 when compared to sBc-arTregs (**Supplementary Figure 2A**), consistent with their higher IFN-γ mRNA expression (**Supplementary Figure 2B**), IFN-γ secretion in culture supernatants (**Figure 5B**), and a trend to a greater percentage of cells producing IFN-γ detected intracellularly using flow cytometry (**Figure 5C**, **Supplementary Figure 3A**). However, IFN-γ-producing cells showed comparable FOXP3 MFI when compared with non-IFN-γ-producing cells from the sDC-arTreg cultures (**Figure 5D**, **Supplementary Figure 3B**), suggesting these arTreg are likely still bona fide Tregs. Additionally, the percentage of IFN-γ^+ cells was lower among sBc- and sDC-arTregs when compared to sBc- and sDC-arTconvs (**Supplementary Figure 3C**). Lastly, almost all sBc- and sDC-arTregs expressed CXCR3 protein (**Figure 5E**, **Supplementary Figure 4**). These data suggest that sBc- and sDC-arTregs may be able to traffic efficiently to sites of T_H1 inflammation and suppress T_H1 responses more effectively than circulating Tregs.

We also examined molecules associated with T_H17-like Tregs. Both sBc- and sDC-arTregs expressed relatively low levels of RORC (**Supplementary Figure 2A**), secreted similar levels of IL-17A (**Figure 5J**) and comprised similar percentages of IL-17A-producing cells (**Figure 5K**, **Supplementary Figure 3A**). The level of IL-17 production was much less compared to arTconvs (**Figure 5J**, **Supplementary Figures 3A, C**). IL-17-producing cells showed comparable FOXP3 MFI when compared with non-IL-17-producing cells (**Figure 5L**, **Supplementary Figure 3B**). CCR6 mRNA expression (**Supplementary Figure 2C**) and percentages of arTregs expressing CCR6 were similarly low (**Figure 5M**, **Supplementary Figure 4**).

We next investigated molecules associated with other specialized T helper cells. sBc- and sDC-arTregs expressed similar levels of mRNA encoding GATA3 and BCL6 as seen in primary Tregs (**Supplementary Figure 2A**). sBc- and sDC-arTregs secreted similar levels of IL-4, IL-5, IL-13 (**Figure 5F**) and IL-10 (**Figure 5N**) and contained similar percentages of IL-4- and IL-10 producing cells (**Figures 5G, O** and **Supplementary Figure 3A**). IL-4 producing cells showed a non-significant trend towards lower FOXP3 MFI compared with non-IL-4-producing cells (**Figure 5H**, **Supplementary Figure 3B**). CCR4 mRNA expression (**Supplementary Figure 2C**) was similar between sBc- and sDC-arTregs, and about 50% of sBc- and sDC-arTregs expressed CCR4 (**Figure 5I**, **Supplementary Figure 4**).

We also examined arTreg expression of tissue-homing chemokine receptors. Lymphoid-homing receptor CCR7 mRNA expression (**Supplementary Figure 2C**) and the percentage of arTregs expressing CCR7 were similar between sBc- and sDC-arTregs (**Figure 5P**). Approximately 60% of arTregs expressed CCR7. 30-40% sBc-arTregs from two responders expressed the gut-homing receptor, CCR9, but the percentages of CCR9 in the other sBc-arTreg cultures and the sDC-arTreg cultures were relatively low (**Figure 5Q**). Levels of CCR9 mRNA were below limit of detection (**Supplementary Figure 2C**).

arTreg Repertoire and Specificity

To compare the clonal composition of the sBc- and sDC-expanded arTreg populations, we performed high-throughput sequencing of the T cell receptor β chain (TCRβ) (**Figure 6**). arTreg sets derived from three responder:stimulator combinations (responder defined as Tregs from one donor, and stimulator defined as sBc and sDC derived from the same donor allogeneic to the Treg donor) were used. For each responder:stimulator combination, 2-4 replicate culture wells were set up in parallel, thus a total of 17 TCRβ sequencing reactions were run (**Table S2**). Surprisingly, the top 100 most frequent unique TCRβ CDR3s from sBc- and sDC-arTregs generated from the same responder:stimulator pairs showed less than 10% overlap in all 3 responders (**Figure 6A**). Similarly, low sharing of total CDR3 reads among top 100 clones, counting repeated sequences, were observed (**Figure 6B**). Morisita and Jaccard distance were then used to quantify the similarity of arTreg populations produced from the same responder: stimulator pair. A distance ratio of 1 suggests no similarity, and a ratio of 0 indicates complete similarity (**Figures 6C, D**). The majority of sBc- *versus* sDC-arTregs comparisons had a ratio very close to 1, suggesting little similarity between the sBc- and sDC-arTreg TCR repertoires.

The low overlap between sBc- and sDC-arTregs' TCR repertoires may be due to expansion of distinct arTreg clones stimulated by sBc and sDC. Alternatively, the primary Tregs at the start of the sBc and sDC cultures may have had distinct repertoire due to limited sampling (100,000 to 250,000/well) of highly clonally diverse circulating Tregs. This latter possibility is supported by the observation that replicate cultures of sBc-arTregs or sDC-arTregs had limited CDR3 overlap and repertoire similarity (**Supplementary Figure 5**). To further test this idea that the narrow sampling of a very diverse pool of Tregs at culture initiation limited repertoire overlap between sBc- and sDC-arTregs., we simulated higher Treg input, thus wider sampling, by digitally pooling replicate wells together to increase the Treg inputs to 200,000 to 750,000/condition (**Table S3**). Two of three responder:stimulator pairs (R1 and R3) had greater sharing between digitally pooled sBc- and sDC-arTreg repertoire compared to individual replicate wells. These data suggest the difference in TCR repertoires stimulated by sBc and sDC is largely due to limited sampling of a very diverse population of blood Tregs.

CDR3 sequences are useful for tracking T cells at the clonal level because they are uniquely generated during T cell development. CDR3 sequences are important determinants of peptide specificity of the TCR. However, since alloreactive TCRs likely interact with the polymorphic frame region of the HLA, not specific to the peptides presented in the HLA (28), the CDR3 sequence may not reflect the alloreactivity of the TCR. Thus, although sBc- and sDC-arTregs use different CDR3 sequences, these differences may not correlate with any differences in their alloreactivity. To compare the alloreactivity of sBc- and sDC-arTregs, we examined their suppressive function stimulated by alloantigens. sBc- and sDC-arTregs showed similar potency in suppressing the proliferation of autologous PBMCs stimulated by irradiated PBMCs from the same donor used to generate the

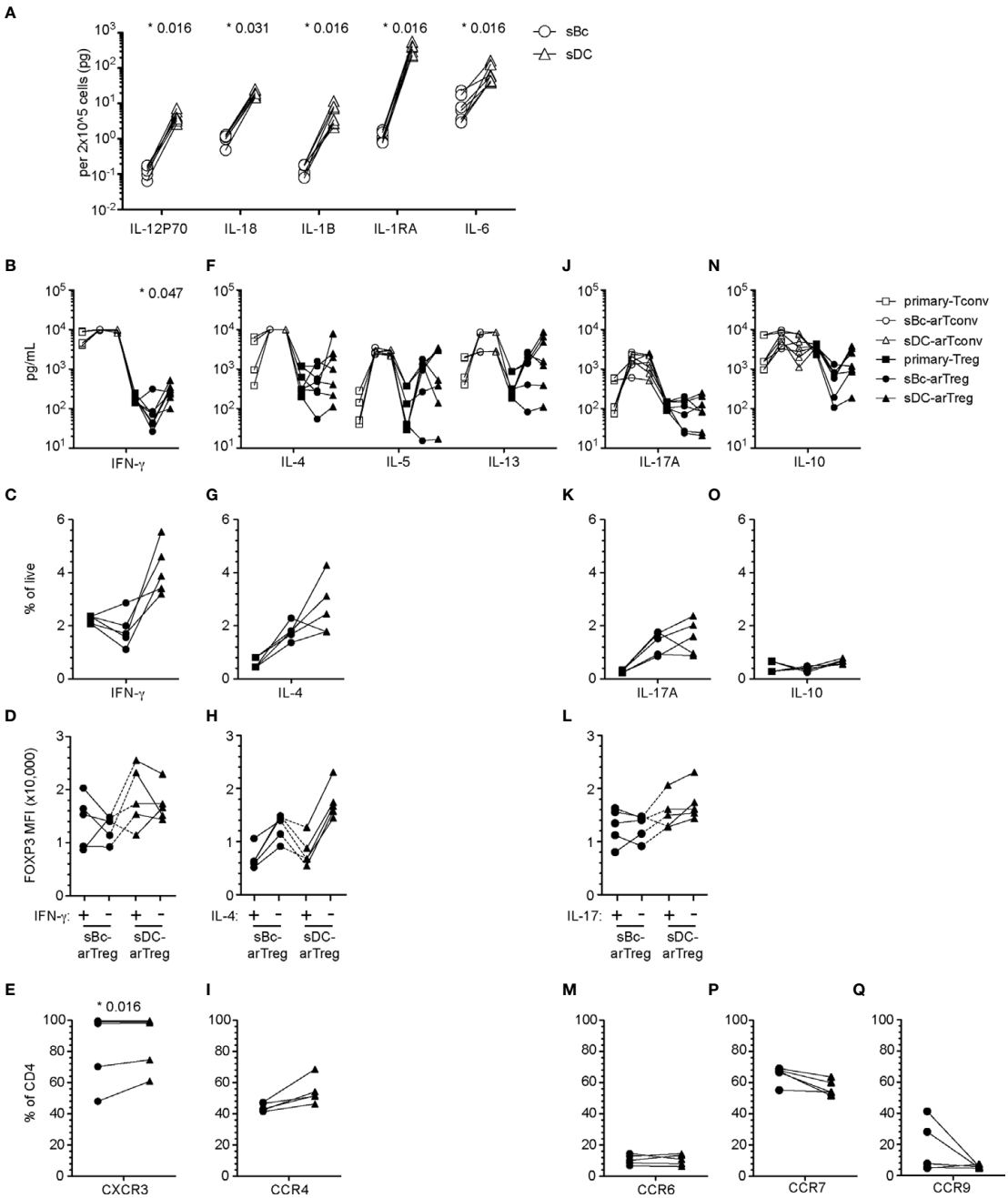


FIGURE 5 | sBc- and sDC-arTregs acquire specialized characteristics while maintaining expression of FOXP3. **(A)** Secretion of cytokines by sBcs and sDCs. sBcs and sDCs were plated alone at the same density used to culture purified T cells (200,000 sBcs or 12,500 sDCs in 100µL assay medium). After 48 h, the culture supernatants were harvested and tested for the indicated molecules using Luminex assay. **(B, F, J, N)** Primary T cells and cultured alloreactive T cells were stimulated with anti-CD3/CD28 beads for 24 h. Supernatants from stimulated cells were collected and analyzed for different cytokines using Luminex assay. **(C, D, G, H, K, L, O)** Primary T cells and cultured alloreactive T cells were stimulated with PMA/Ionomycin in the presence of Brefeldin A and monensin for 5 h. Cytokine production by arTregs **(C, G, K, O)**, and level of expression (MFI) of FOXP3 in cytokine- and non-cytokine-producing arTregs **(D, H, L)** was assessed by intracellular staining. **(E, I, M, P, Q)** Chemokine receptor expression on arTregs. Data in **(A)** contain 7 different sBc and sDC pairs. Connecting lines indicate sBcs and sDCs derived from the same donor. Data in **(B, F, J, N)** contain 7 different responder:stimulator pairs. Data in **(C, D, G, H, K, L, O)** contain 5 different responder:stimulator pairs. Data in **(E, I, M, P, Q)** contain at least 5 different responder:stimulator pairs. Connecting lines indicate alloreactive T cells stimulated by APCs (sBcs or sDCs) derived from the same donor. Statistics in **(A)** were performed using the Wilcoxon matched-pairs signed test. Statistics in **(B, C, E, F, G, I, J, K, M, N, O, P, Q)** were performed comparing sBc- and sDC-arTreg populations using the Wilcoxon matched-pairs signed rank test. Data from primary Tconvs and arTconvs are shown as a reference and were not included in statistical analyses. Statistics in **(D, H, L)** were performed compared cytokine-producing and non-producing cells within the same APC stimulation group using the Wilcoxon matched-pairs signed rank test.

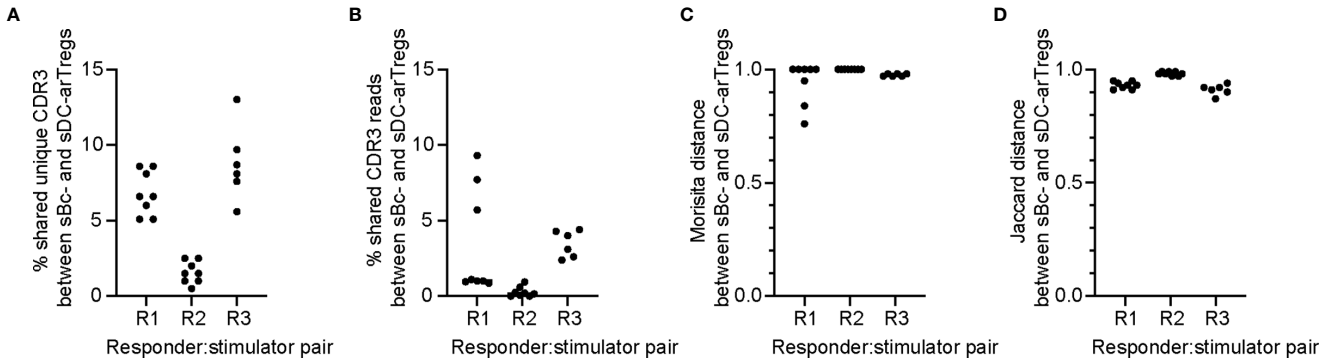


FIGURE 6 | sBc- and sDC-arTregs stimulate distinct TCR repertoire likely in part due to diversity of the circulating Tregs. On d0, 2-4 replicate culture wells were set up using the same responder:stimulator combinations (R1, R2, and R3). Cytokine-matured monocyte-derived DCs were used to stimulate responder 1 and 2, and MPLA-matured monocyte-derived DCs were used to stimulate responder 3. On d11, RNA was isolated from the arTregs in each well (~500,000). RNA from ~250,000 cells was TCRβ sequenced with a read depth of 1 million. For each responder:stimulator combination, the top 100 CDR3s between sBc- and sDC-arTregs were compared. All possible sBc- and sDC-arTreg combinations within the same responder: stimulator pair were compared. Each comparison is represented by one dot. **(A)** Percentage shared unique CDR3, **(B)** percentage shared CDR3 reads, **(C)** Morisita distance comparing CDR3 usage, and **(D)** Jaccard distance comparing CDR3 usage were calculated between sBc- and sDC-arTregs.

sBcs and sDCs (**Figure 7**, left). sBc- and sDC-arTregs exhibited enhanced suppressive activity against proliferation stimulated by relevant donor APCs than by an unrelated donor (**Figure 7**, right). Overall, sBc- and sDC-arTregs appear to have similar alloreactivity and have similar potent and specific suppressive function.

DISCUSSION

Previous studies have shown that use of sBcs as APCs is an effective way of expanding Tregs (12, 29). sBc-arTreg manufacturing process has already been reviewed by the FDA in the context of several ongoing early phase clinical trials. Stimulatory DCs are potent APCs and may be a viable alternative for manufacturing clinical grade arTregs. In this study, we compared the stimulatory capacity of sBc and sDC to expand arTregs, and characterized the *in vitro* characteristics of these expanded arTregs.

In general, we found that sDCs, versus sBcs, led to 2-fold more arTreg expansion, which may be due to sDCs' increased expression of CD80 and CD86 and chemokines. However, Tregs from a few responders showed comparable or less proliferation when stimulated with sDCs than with sBcs. These results are likely due to certain undefined responder-Treg characteristics, because the sDCs used in these experiments were more potent stimulators of other responder Tregs compared the sBcs (data not shown). Future *in vitro* studies using blocking antibodies against co-stimulatory molecules, chemokines, and/or other

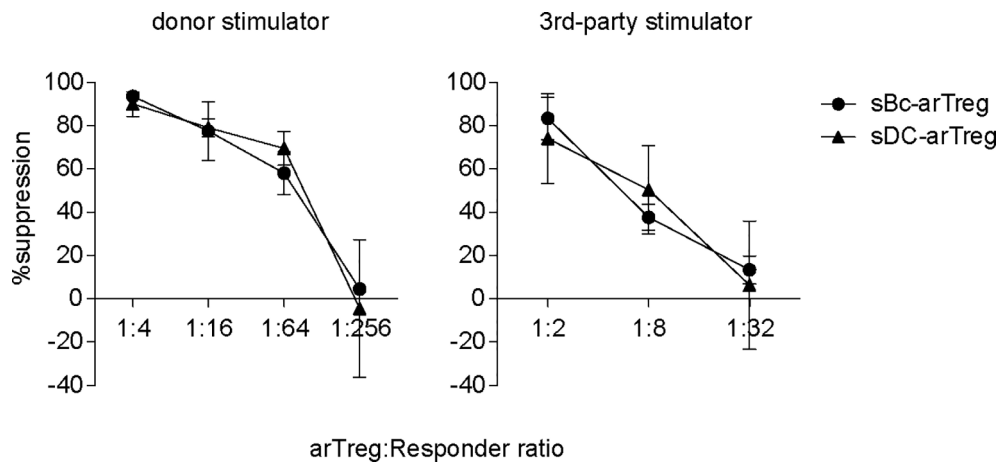


FIGURE 7 | Both sBc- and sDC-arTregs suppress T cell proliferation. Suppression of autologous PBMC proliferation by sBc- and sDC-arTregs that were stimulated from APC donor (left) or from third-party donor (right). Data contain n=2 (donor) and n=3 (third-party) different responder-stimulator combinations. Statistics were performed using unpaired t-test between sBc-arTregs and sDC-arTregs at the same Treg dilution.

soluble factors can be performed to dissect the mechanisms on how sDC stimulate more arTreg expansion. Also, it would be interesting to determine whether cell-to-cell contact between the sDC and Tregs is necessary for increased proliferation.

We found that sBc- and sDC-arTregs are very comparable in purity, phenotype, antigen-specific suppression. Although sBc- and sDC-arTregs expressed similar levels of Treg-associated molecules, we found 23 genes that were differentially expressed by at least 2-fold between sBc- and sDC-arTregs. Further mechanistic studies can use blocking antibodies to some of these proteins to see whether they affect proliferation of Tregs or alter Treg suppression capability.

One potential concern with using sDCs is that they may produce higher levels of pro-inflammatory factors that could destabilize Tregs. We found higher IL-1β, IL-6, and IL-12p70 expression by sDC than by sBcs. However, the levels of these cytokines were very low in both cultures. More importantly, sBc- and sDC-arTregs had similar percentages of FOXP3 enhancer demethylation, similar phenotypes and suppressive functions, suggesting that neither stimulatory APC type induced Treg destabilization during the selective expansion of arTregs. Low percentages arTregs expressed IFN-γ, IL-4, and IL-17 and most of these cytokine-producing cells were $FOXP3^+$, whereas most sBc- and sDC-arTregs expressed CXCR3. Together, our phenotype analyses show that both sBc- and sDC-arTregs have a stable, committed Treg phenotype and may have enhanced ability to traffic to sites of T_H1 inflammation, such as transplanted organs undergoing alloimmune attack. Results from this study of direct comparison between sBc and sDC showed that arTregs expanded by these 2 APCs were, for the most part, are comparable in terms of purity, phenotype, and antigen-specific suppression.

Our previous study demonstrated that sBc-arTregs were effective *in vivo* in preventing alloimmune-mediated injury of human skin allografts (12). sBc-arTregs were able to home to transplanted skin allografts and were detected 6 weeks after injection into mice. In this current study, we found sBc- and sDC-arTregs to be phenotypically and functionally similar. The cells have similar high demethylation of the FOXP3 enhancer. Thus, we speculate that sDC-arTregs would have similar suppressive activity, comparable stability, and migration patterns *in vivo* as demonstrated previously with sBc-arTregs.

Currently, four registered clinical trials (NCT02188719, NCT02244801, NCT02474199, NCT02711826) are using sBcs as stimulatory APCs to generate arTregs. Using sDCs to manufacture arTregs could potentially provide some key advantages over using sBcs. First, arTregs expand 2-fold more after stimulation with sDCs than with sBcs. Second, sDC differentiation and maturation from monocytes takes 7 days, which may be further shortened (30), whereas sBc generation takes at least 10 days. Third, sDC cultures require minimal handling before harvesting, whereas sBc cultures require regular feeding and restimulation. Fourth, B cell stimulation with CD40L requires feeder cells. Although CD40L stimulation of B cells can be achieved without feeder cells by using a cell-free soluble 4-trimer CD40L reagent (UltraCD40L) (31), this reagent may not be widely available as a GMP reagent, whereas sDC production can be feeder-free and rely solely on well-defined soluble GMP-grade reagents. Fifth, B cells, not monocytes, harbor latent Epstein Barr Virus (EBV). Stimulating B cells to sBcs can potentially lead to the reactivation of latent EBV. Detection of infectious EBV in sBcs will lead to termination of clinical arTreg manufacturing. Sixth, less sDCs are required to stimulate T cells compared to sBcs. One disadvantage of using sDCs is that they do not increase in number during *in vitro* differentiation and maturation, whereas sBcs can expand more than 10-fold during 10-day culture. Despite the need for less sDCs to expand Tregs, more donor blood will be needed to manufacture sDCs. Also, spleen from the donor is commonly available to make donor sBcs. While sBcs can be generated from splenocytes, it remains to be demonstrated that splenic $CD14^+$ monocytes can be differentiated into sDCs. Previous studies have shown precursors in mouse spleen can be cultured to develop into stimulatory DCs (32–34). Another potential source of monocytes from human donors is bone marrow cells. Taking in these considerations of advantages and disadvantages of using sDCs *versus* sBcs, sDCs are slightly better for their relative simpler culturing process and slightly better Treg expansion.

Together, our results show that sDCs have more potent Treg expansion ability and the resulting arTregs are similar to those expanded with sBcs. We propose that sDCs may be a viable alternative to manufacture arTregs for clinical use.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UCSF (Study 10-01980) and University of Pittsburgh (Study 19120084) institutional review board approval. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LL: study design, data generation and analysis, and writing of the manuscript. HZ: study design, data generation and analysis, and manuscript review. KL: study design, data generation, and manuscript review. HL: data generation. AM and EM: data analysis and interpretation. FV: manuscript review. AT: study design, and manuscript review. QT: research design, data analysis, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.679675/full#supplementary-material

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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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REVIEW
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The Role of Regulatory T Cells in Epicutaneous Immunotherapy for Food Allergy

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In recent decades, a rapid increase in the prevalence of food allergies has led to extensive research on novel treatment strategies and their mechanisms. Mouse models have provided preliminary insights into the mechanism of epicutaneous immunotherapy (EPIT)-induced immune tolerance. In EPIT, antigen applied on the skin surface can be captured, processed, and presented in the lymph nodes (LNs) by Antigen-presenting cells (APCs). In the LNs, induction of regulatory T cells (Treg cells) requires both direct contact during antigen presentation and indirect mechanisms such as cytokines. $Foxp3^{+}CD62L^{+}$ Treg cells can exhibit the characteristics of hypomethylation of Foxp3 TSDR and $Foxp3^{-}LAP^{+}$ Treg cells, which increase the expression of surface tissue-specific homing molecules to exert further sustained systemic immune tolerance. Studies have shown that EPIT is a potential treatment for food allergies and can effectively induce immune tolerance, but its mechanism needs further exploration. Here, we review Treg cells' role in immune tolerance induced by EPIT and provide a theoretical basis for future research directions, such as the mechanism of EPIT and the development of more effective EPIT treatments.

Keywords: allergen-specific immunotherapy (AIT), epicutaneous immunotherapy (EPIT), food allergy, regulatory T cell (Treg cell), immune tolerance

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INTRODUCTION

Food allergies are a growing concern given their increasing global incidence in recent decades. WHO has listed it as one of five major public health problems (1). Research shows that food allergies are more common in developed countries, and up to 8% of children and 5% of adults in Western countries suffer from food allergies (2, 3). Besides, the incidence of food allergies in children is higher than that of adults. Epidemiological surveys have shown that up to 4.5% to 13.5% of children in Japan suffer from food allergies (4).

Strictly avoiding allergenic foods after correct diagnosis and preparing for adrenaline injection in accidental exposure cases is still the most effective therapy at present. However, some common allergenic foods, such as milk and eggs, are commonly used as food additives in processed foods. In addition, food allergen information labeling is not perfect, and it is difficult to avoid altogether accidental food allergen consumption, which is challenging for patients and their families (5). To date, studies related to food allergies have mainly focused on allergen-specific immunotherapy

(AIT), DNA vaccines, dietary supplements, Chinese herbal formulae, adjuvant-enhanced immunotherapy, and the introduction of allergenic foods in the early stages of life (6, 7). AIT is a potentially effective treatment for food allergies, and its effectiveness and safety have been confirmed to some extent (8, 9). However, the specific mechanism needs further research.

The ultimate goal of AIT treatment is to enable patients to tolerate allergens, and various types of immune cells play an important role in this process. Accumulating evidence indicates that AIT may act by modifying the patterns of cytokines produced by helper T cells (Th) (10). Researches of mice and humans have identified Th2 cytokines [interleukin (IL)-4, IL-13, and IL-5] as major contributors to allergic disease (11–13), while AIT can bias the immune response of allergic patients to Th1 type immune response. Majori et al. (10) found that AIT has a significant effect on increasing the interferon-γ (IFN-γ, Th1 cytokine)/IL-4 ratio in peripheral blood $CD4^+$ T cells, which is consistent with the observations of Ohashi (14) and Varney (15). Besides, Hamid et al. (16) showed that IL-12 can effectively stimulate the proliferation of Th1 T lymphocytes in AIT treatment, and IL-12 may inhibit late-phase responses after successful immunotherapy. In addition, AIT induced antigen-specific suppressive activity in $CD4^+CD25^+$ T cells of allergic individuals, which is in line with the immunosuppressive effect observed in non-allergic individuals, was observed in Jutel's research (17). More specifically, the deviated immune response was characterized by suppressed proliferative T cells and Th1 and Th2 cytokine responses, and increased IL-10 and TGF-β secretion by allergen-specific T cells (17). Additionally, IL-10-producing regulatory B cells (Breg cells) also showed a potent suppressive capacity on antigen-specific $CD4^+$ T-cell activation (18). Breg cells were observed most abundantly within the initial weeks of immunotherapy and gradually returned to baseline as AIT progressed (19). However, regulatory T cells (Treg cells) were observed in the later stages of AIT, indicating the role of Treg cells in the induction of long-term immune tolerance by AIT (20). Moreover, the critical role of Treg cells in various types of AIT has been further confirmed in mouse models (21, 22). Here, we review Treg cells' role in immune tolerance induced by epicutaneous immunotherapy (EPIT) and provide a theoretical basis for future research on the mechanisms of EPIT and the development of more effective EPIT treatments.

REGULATORY T CELLS

In 1971, Gershon and Kondo (23) first discovered a subset of T cells pretreated with thymocytes that may have immunosuppressive effects and prevent otherwise 'helpful' T cells from mediating heir function. Subsequent studies had also confirmed the existence of such suppressor T cells (Ts cells) (24–32). However, the development of Ts cells suffered a major blow in the early 1980s (33). Biochemical and molecular experiments questioned the interpretation of earlier studies, and the term "suppressor T cell" almost disappeared within a few years (33–35). As Darwin said, science is to sort out the facts, so that from the common rules and conclusions. For about 30 years, scientists had not given up on the study of Ts cells, they had to cover up their data in the name of "down-regulation" or "infectious tolerance or anergy". As Ronald described, like a phoenix, negative regulatory T cells rose from these ashes to a position of prominence in today's immunological thinking over precisely the interval from the demise of Ts to the present (33). Fortunately, since the late 1990s, the interest in Ts cells was reborn. In 1995, Sakaguchi et al. (36) discovered a subset of thymus-derived $CD4^+$ T cells that continuously express CD25, the receptor α chains of IL-2, which can protect thymectomized mice from autoimmunity and was later named Treg cells (37). Since then, substantial researches have explored the immunosuppressive effects of Treg cells and their mechanisms. Apart from protecting from autoimmunity, Treg cells also play a role in other pathological and physiological immune responses, such as allergy (38), tumor immunity (39, 40), transplantation (41, 42), and microbial immunity (43, 44), and can also be targeted to suppress or enhance the immune responses in clinical settings (45).

Different studies showed that the expression of the transcription factor Foxp3 faithfully identifies these naturally occurring Treg cells (46–49). Moreover, loss-of-function mutations of the *Foxp3* gene lead to poor development of $CD4^+CD25^+$ Treg cells (47, 48). These findings together led people to believe that $Foxp3^+$ Treg cells represent a stable cell lineage. Subsequent studies began to use Foxp3 as a "specific" molecular marker for Treg cells to reveal the molecular and cellular mechanisms of Treg cell differentiation and function (50). However, *Foxp3* alone does not control all aspects of Treg biology and is not the initiating factor in Treg development. A fact made clear as $CD25^+Foxp3^-$ Treg precursors in the thymus are already fate committed to the Treg cells lineage despite their lack of Foxp3 expression (51–54). Importantly, induction and maintenance of Foxp3 expression are two separable processes regulated by distinct *cis*-regulatory elements within the *Foxp3* locus (55). Treg cell-specific demethylation region (TSDR), that is, the region where the cytosine-guanine dinucleotide (CpG) site in Treg cells is completely demethylated, has been shown to be required for heritable maintenance of the stable and high expression of Foxp3 in dividing Treg cells and play a key role in Treg cells' inhibitory function (55, 56). Furthermore, TSDR was thought to identify the "real" human Treg cells (57). Besides, Treg cells also express surface molecules such as costimulatory molecules CD28 (58), chemokine receptors CCL27/28 (CCR10) (59), CCL20 (CCR6) (60), and CCL17/22 (CCR4) (61).

In the literature, Tregs are divided into subpopulations according to differentiation sites and the expression of well-known functional markers. For the first time in 2009, Battaglia et al. (62) divided human Treg cells into three subgroups according to the expression levels of CD25, CD45R, and Foxp3: $CD25^{++}CD45RA^+$ ($Foxp3^{lo}$) resting Treg cells (rTreg cells), $CD25^{+++}CD45RA^-$ ($Foxp3^{hi}$) activated Treg cells (aTreg cells), and $CD25^{++}CD45RA^-$ ($Foxp3^{lo}$) cytokine-secreting T cells. Of these, rTreg cells represent naive Treg cells, and aTreg cells represent effector Treg cells at different differentiation stages. The former two subgroups exert immunosuppressive functions, while

cytokine-secreting T cells lack inhibitory activity (62). Despite the improvement in Treg cells biology, there are no specific markers to characterize human Treg cells, and the expression of Treg cell surface molecules is not constant. This classification still has certain limitations. Also, they can be further characterized by the site of differentiation: thymus-derived Treg cells (tTreg cells), peripherally induced Treg cells (pTreg cells), and *in vitro* induced Treg cells (iTreg cells) (63). tTreg cells usually include rTreg cells and aTreg cells (64). pTreg/iTreg cells are generated from conventional $Foxp3^{-}CD4^{+}$T cells, and the expression of Foxp3 can be induced after IL-2, retinoic acid, and TGF-β activates CD3 signaling molecules (63). However, a phenotypic distinction between tTreg cells and pTreg cells has not yet been established (65). Accumulating evidence indicates that tTreg cells and pTreg cells play different roles in different tissues. tTreg cells persist in the periphery, play a stable function role in maintaining dominant self-tolerance (45). Besides, due to the nature of pTreg cells differentiation (non-self-antigens and a particular TCR signaling combined with other signals), these cells are assumed to be more functional for maintaining mucosal tolerance (66–68).

Studies have shown that Treg cells' level in children with food allergies is significantly downregulated, leading to a decline in immunosuppressive function. This decline can promote and aggravate allergies, which illustrates the critical role of Treg cells in maintaining immune tolerance in the body (69). As shown in **Figure 1**, Treg cells can directly exert immunosuppressive functions in several ways (67, 68): 1) secretion and production of IL-10, TGF-β, IL-35, as well as granzyme and perforin, which directly affect the proliferation, activation, and apoptosis of conventional T cells (Tcons) (70). Treg cells can inhibit the proliferation of Th1 and Th2 cells and the secretion of cytokines (such as Th1 cytokines: IL-2, IFN-γ; Th2 cytokines: IL-4, IL-5, and IL-13), they also can promote Th17 cells proliferate and secrete IL-17 to exert an inhibitory effect (68). At the same time the induction of high affinity effector and memory $CD8^{+}$ T cells is reduced (67); 2)Treg cells can affect the proliferation, activation and apoptosis of B cells in the manner described in 1); 3) inhibition of TCR-induced Ca^{2+}, NFAT, and NF-κB signaling in Tcons, and inhibition of B cells through the PDL1/PD-1 signaling pathway (51); 4) direct inhibition of the proliferation and effect of NK through membrane-bound TGF-β, mainly through inhibition of the expression of the latter's

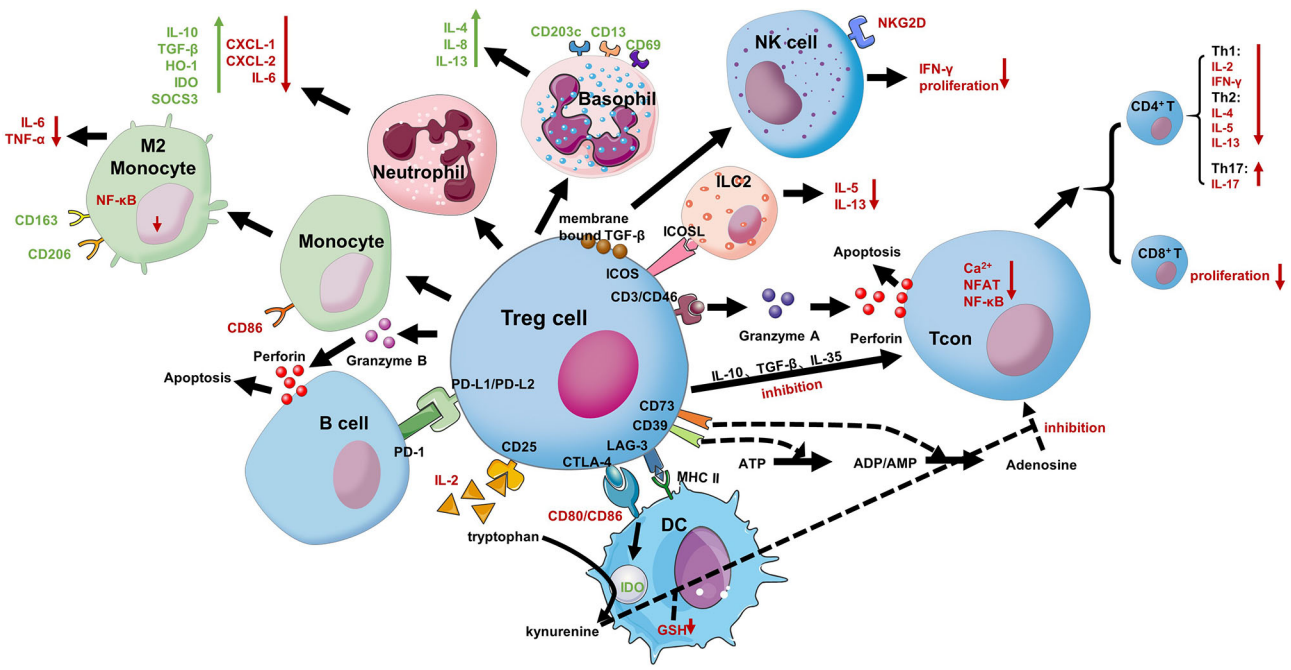

FIGURE 1 | Treg cells suppressive mechanisms. Treg cells inhibit the proliferation and effects of NKs through membrane-bound TGF-β, mainly including inhibiting the expression of the latter's surface protein NKG20 and the production of IFN-γ. Treg cells can inhibit the secretion of IL-5 and IL-13 by ILC2 in an ICOS/ICOSL-dependent manner, thereby inhibiting its function. Treg cells can inhibit conventional T cells (Tcons) action in many ways. 1) Treg cells can produce anti-inflammatory cytokines (IL-10, IL-35, and TGFβ) affecting Tcons; 2) they can release perforin and granzyme, which damage the target cell membrane leading to apoptosis; 3) Treg cells can also sequester, by the high expression of CD25, IL-2 from the microenvironment reducing effector Tcons proliferation; 4) Treg cells can quickly inhibit TCR-induced Ca^{2+}, NFAT, and NF-κB signaling; 5) indirectly inhibiting Tcons by reducing the expression of CD80/CD86 on DCs through CTLA-4 (inhibit DC antigen presentation function) or disrupting the microenvironment in the immunological synapse provided by DCs (essential for T cell proliferation); 6) The expression of CD39 on Treg cells mediates the conversion of ATP to adenosine and reduces the proliferation of Tcons. Treg cells can directly affect B cells *via* PDL1/PD-1 interaction and DCs *via* CTLA-4 and LAG-3. CTLA-4 blocks co-stimulation, reducing CD80/CD86 expression, and it induces upregulation of IDO. Treg cells can inhibit B cells action and release granzyme B and perforin through the PD-1 signaling pathway to kill B cells. Treg cells can also bias monocytes to M2 macrophages, enhancing CD163 and CD206 on their surface molecules. They can similarly induce the suppressive phenotype of neutrophils and basophils and reduce the secretion of ILC2 cytokines.

surface protein NKG20 and the production of IFN-γ (71, 72); 5) inhibition of IL-5 and IL-13 secretion by ILC2s in an ICOS/ICOSL-dependent manner, thereby inhibiting its function (73); 6) direct inhibition of DCs through both the CTLA-4/CD80 and LAG-3/MHC II signaling pathways (68, 74); 7) acting on monocytes and granulocytes, inhibiting their cytokine secretion, differentiation, and antigen-presenting function (75, 76). Besides, Treg cells can act indirectly by 1) highly express CD25 to create a microenvironment lacking IL-2 so that Treg cells can "starve" surrounding cells that need this cytokine (77); 2) promoting extracellular ATP conversion into adenosine and AMP, which have immunosuppressive effects, by expressing CD39/CD73 (70); 3) indirectly inhibiting Tcons by reducing the expression of CD80/CD86 on DCs through CTLA-4 (inhibit DC antigen presentation function) or disrupting the microenvironment in the immunological synapse provided by DCs (essential for T cell proliferation) (67, 78).

ALLERGEN-SPECIFIC IMMUNOTHERAPY FOR FOOD ALLERGY

AIT is considered the only treatment for allergic diseases that can effectively change the disease's course. Its efficacy for allergic asthma, rhinitis, and allergic diseases has been confirmed (79–81). In recent years, AIT has been used to treat food allergies and is considered a potentially effective treatment for allergic diseases (82–84). The principle of AIT is to gradually increase the allergen dose to reduce the patient's responsiveness to allergenic foods and ultimately achieve the goal of desensitization and sustained unresponsiveness (85).

AIT includes subcutaneous immunotherapy (SCIT), oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and EPIT. Researches on SCIT for food allergies have shown that it can cause severe side effects, so it is generally not considered a treatment (86, 87). OIT is currently the most widely studied method for the treatment of food allergies. Studies have shown that OIT can effectively treat egg (88), milk (89, 90), and peanut (91, 92) allergies. It is worth noting that the world's first approved food allergy treatment drug, PALFORZIA, was approved by the US Food and Drug Administration (FDA) to treat peanut allergy patients on January 31, 2020. Although clinical studies of PALFORZIA have shown that this oral immunotherapy can lead to rapid desensitization to peanut protein and improve allergy sufferers and their guardians' quality of life, almost all participants reported adverse events (93). In addition, Chu et al. (94) systematically evaluated the potential risks of peanut OIT treatment. The results showed that, comparing with avoiding peanuts, the risk of allergic reactions during peanut OIT treatment increased 3.12 fold, and the risk of using epinephrine increased 2.21 fold. SLIT is used as a potential alternative to OIT. SLIT involves the administration of small drops of allergen extract (micrograms to milligrams) under the tongue for approximately 2 minutes, which is then eventually spit or swallowed (95, 96). During this process, it is absorbed by Langerhans cells (LCs) and is finally brought into the draining lymph nodes (dLNs) to induce antigen-specific tolerance (97). The dosage of SLIT is 1/100-1/1000 of OIT. The secondary effects of SLIT are mainly itching and oropharyngeal irritation. Although most studies have reported systemic adverse reactions, they are not common compared with OIT (98, 99). However, its effectiveness needs to be further explored.

EPICUTANEOUS IMMUNOTHERAPY

In addition to injection or oral administration, the skin is also a promising treatment site for diseases. As a target area for treatment, skin has many advantages. First, as a non-vascularized tissue, the epidermis can strictly restrict the entry of allergens into the bloodstream, minimizing the risk of acute side effects. Second, skin is rich in antigen-presenting cells (APCs), especially immunomodulatory macrophages (100). Therefore, allergens can be transported in the intact skin, and by activating APCs, it can further promote the production of allergen-specific Treg cells, which can prevent and treat food allergies (101, 102). Third, compared with the oral route, applying the drug to the skin can prevent the substance from being chemically or enzymatically disintegrated in the gastrointestinal tract or liver, which is especially important for protein or peptide drugs that are widely used in immunotherapy, such as insulin (103) and antibody (104). Last but not the least, skin is a more accessible treatment site to manage, which provides convenience for patients to treat themselves at home. These advantages have aroused people's interest in its development. In addition, as a skin application method, transdermal patches can be traced back to ancient China (around 2000 BC), when people began to apply medicated plasters containing multiple herbal ingredients to the skin as a treatment method (105, 106). With trial, error, clinical observation and evidence-based studies, transdermal patches are now widely used as cosmetic, topical and transdermal delivery systems (105). The studies of dermal application induced suppression were inspired by earlier observations showing that epicutaneous application of protein antigen on the skin in a form of a gauze dressing induces the synthesis of IL-4 and IL-13, which may potentially inhibit the immune response mediated by $CD4^+$ Th1 lymphocytes (107, 108). Later more studies were conducted on the immunosuppressive effects of EPIT. Mouse models of contact sensitivity (CS) (109, 110), experimental autoimmune encephalomyelitis (EAE) (111), collagen-induced arthritis (CIA) (112), and colitis (113) showed that, the epicutaneous application mainly exerted an inhibitory effect by inducing Ts/Treg cells, rather than simply suppressing Th1 type response or Th2 type immune response.

EPIT, involving transdermal administration of allergen under an occlusive dressing that promotes allergen absorption, was introduced as a treatment for allergies surprising early (114, 115), and has gradually been used to treat food allergies due to the increase prevalence. To date, EPIT's researches on food allergy treatment mainly focus on the egg (ovalbumin, OVA), milk (116, 117), and peanut allergies. EPIT usually consists of the daily application of a new patch on designated skin locations for

maintenance dosing, involving cutaneous exposure to micrograms of allergens. OIT protocols start with an initial dose-escalation phase and then the maintenance phase. However, unlike the OIT protocol, the patch's allergen content remains constant during EPIT treatment, but the daily application time of the patch gradually increases. What's more, the most commonly used product in research is a product called Viaskin® (DBV Technologies, Bagneux, France). The Viaskin® epidermal delivery system (EDS) forms an occluded chamber on the skin that generates moisture and releases allergen proteins from its support. The protein is then absorbed through the skin, where it interacts with epidermal immune cells (118). Researches show that the product has sound therapeutic effects in mouse models and patients with a peanut allergy aged 4-11. Another key fact to notice is that there are no reports of severe side effects in clinical studies, indicating high safety (118–121). Viaskin® has now completed Phase III clinical trials (122) and is currently undergoing a five-year Open-Label Extension PEPITES study (PEOPLE) (123). The objectives who have now completed three years of active treatment in PEOPLE demonstrate that daily EPIT treatment for peanut allergy beyond one year leads to a continued response from a well-tolerated, simple-to-use regimen. Although Viaskin® received the FDA's Breakthrough Therapy Designation (BTD) in 2015, it is still under review and has not been approved for use or sale in any country/region. To be approved, like PALFORZIA, more clinical trials are needed, including larger cohort and more extended durations studies. It is necessary to obtain more data that can be used to evaluate and support the overall risk/benefit relationship related to the Biologics License Application (BLA), including the safety, efficacy, effective treatment dosage and expected treatment endpoints of Viaskin® (124).

The skin is an active immune organ, in which the microbiome, chemical, physical and immune barriers form an interactive network that can prevent the invasion of foreign proteins and peptides and other macromolecules (125). Although this protective effect of the skin plays an important role in maintaining the body's immune function, this protective effect will also limit the dose of allergens or drugs delivered to the skin during EPIT treatment, which greatly limits the therapeutic effect (83, 118). Therefore, the skin application of proteins and peptides may still be a challenge. For example, studies have shown that the delivery efficiency of Viaskin® EDS is only about 10% (126), which may be the main reason for the poor therapeutic effect of this product. To improve drug or vaccine delivery, tape stripping (to remove of epidermis corneal layer) (127), the use of liposomes (128, 129), niosomes (130), and membranes equipped with microneedles (131) have been applied to increase the skin permeability.

In addition, in order to improve the efficiency of allergen delivery in EPIT treatment, Kumar et al. (132) applied a patch containing allergens and adjuvants [1,25-dihydroxyvitamin D_3 (VD3) and CpG oligodeoxynucleotides (CpG-ODNs)] to the back skin of OVA-sensitized mice pretreated by ablation micro-fractional laser, which is called μEPIT here. The research results show that μEPIT can deliver 80% of the powder in the patch into the mouse within about 1 hour, a faster and more efficient EPIT treatment. It is also worth mentioning that CpG may be a good adjuvant of EPIT. As a monotherapy, an adjuvant or an ingredient of vaccines, animal experiments have proven its effect in infectious diseases, allergies, and oncological diseases (133). CpG can be administered by injection, inhalation, oral, or even vaginal routes, but the safety of various administration methods is still controversial. In the clinical trials of Peter et al. (134), a group of patients with hay fever showed that subcutaneous administration of allergen with CpG alleviates clinical symptoms in comparison with the placebo group. However, CpG injection often leads to many local and systemic adverse reactions, the intensity of which depends on the CpG dose (133). Local symptoms comprise pain, skin flushing, edema, and pruritus, moreover, systemic symptoms are more severe, which include headache, myalgia, fever, nausea, and vomiting (133). It is worth noting that compared with other routes, such as subcutaneous injection, epicutaneous administration of CpG seems to be safer and may not give side effects. Majewska-Szczepanik et al. (135) found that epicutaneous application of CpG with OVA antigen inhibits atopic dermatitis in mice. More interestingly, epicutaneously applied CpG was not absorbed and was not detectable in serum, indicating higher safety. The combination of CpG and ODN as an adjuvant has been confirmed to some extent, but determining a more effective combination of CpG may be one of the development directions of EPIT adjuvants in the future. Besides, to tailor this powder delivery technology for clinical uses, Wu's team (136) developed a powder-laden dissolvable microneedle array (PLD-MNA) that can carry antigen powder for EPIT. Their research results confirmed that the PLD-MNA antigen presentation rate is close to 100%. This novel, safe, effective, and self-managed food allergy treatment method is expected to become a new food allergy EPIT method. In addition to destroying the skin barrier, Sayami et al. (137) also tried to improve the patch material to promote antigen presentation. They developed an allergen-containing hydrophilic gel (HG) patch to treat milk allergy. The protein layer formed on HG surface creates a concentration gradient that becomes the force driving protein penetration, thereby improving antigen delivery efficiency (138). More importantly, a milk-sensitized mouse model and clinical trial have confirmed this EPIT patch's therapeutic effect on milk allergy (137).

THE ROLE OF TREG CELLS IN EPIT

In 2011, Dioszeghy et al. (101) used Viaskin® EDS loaded with OVA for EPIT treatment and analyzed the systemic cellular immune response of EPIT. They found that the percentage of $CD25^+Foxp3^+$ CD4 T cells in the spleen of EPIT-treated mice was significantly higher than those of the sham group. Later in the researches of Mondoulet et al. (139, 140) also showed that the mRNA expression of Foxp3 in the EPIT group was significantly higher than that of the control group. These studies all

underlined the involvement of Treg cells in EPIT. In 2014, Dioszeghy et al. (141) further explored the role of Treg cells in EPIT. They first used Viaskin® EDS loaded with peanut allergen to treat peanut-sensitized mice for eight weeks with or without anti-CD25 antibodies injection. Moreover, they found that EPIT significantly increased the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen of peanut-sensitized mice. However, the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen was lowered with the intraperitoneal injection of anti-CD25 antibody. Consequently, the EPIT treatment effect was inhibited at the system level, indicating the role of $CD4^+CD25^+Foxp3^+$ Treg cells in the induction of immune tolerance by EPIT. In addition, they also transferred the $CD4^+CD25^+$ Treg cells induced by EPIT treatment to peanut-sensitive mice or Foxp3-IRES-mRFP mice, respectively, and determined the maintenance of Treg cells after EPIT termination and the ability to induce host Treg. In their study, both $Foxp3^+CD62L^+$ and $Foxp3^+CD62L^-$ Tregs increased significantly following EPIT. Yu et al. (136) used a PLD-MNA to treat mice with EPIT and analyzed $CD4^+$ T cells in the spleen and LNs using flow cytometry. The results also confirmed that EPIT effectively induced $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen and LNs.

Moreover, to determine the $Foxp3^+$ Treg cell subtypes that play a role in EPIT treatment, some studies have analyzed the expression of CD62L on its surface. CD62L is a marker utilized to distinguish naive cells from effector cells, is a crucial lymphoid homing molecule. After EPIT treatment of milk-allergic mice with Viaskin® EDS containing milk, Mondoulet et al. (142) collected $CD4^+CD25^+Foxp3^+CD62L^+/CD62L^-$ Treg cells from the mouse spleen. They transferred them to unsensitized mice before initiating peanuts. By measuring allergic indicators such as body temperature and mast cell protease-1 levels in mice in each group, it was found that compared with the positive control group, only mice in the $CD4^+CD25^+Foxp3^+CD62L^+$ Treg cells transfer group were protected from allergic reactions. This result indicated that $CD4^+CD25^+Foxp3^+CD62L^+$ Treg cells might play an essential role in the induction of immune tolerance through EPIT treatment. Furthermore, $CD4^+CD25^+$ $Foxp3^+CD62L^+$ Treg cells have been proved to be a Treg subtype with strong immunosuppressive effects that can prevent the occurrence of fatal acute graft-versus-host disease (GVHD) (143). Dioszeghy et al. (144) compared the phenotype and inhibitory activity of Treg cells induced by EPIT, OIT, and SCIT in peanut-sensitive mice. They found that a significant difference in the phenotype of EPIT-induced Treg cells was the induction of both effector/memory ($Foxp3^+CD44^{hi}$ $CD62L^-$) Treg cells and naive ($Foxp3^+CD44^{lo}CD62L^+$) Treg cells. In contrast, OIT and SLIT induced only effector/memory Treg cells. Moreover, whereas OIT- or SLIT-induced Treg cells lost their suppressive activity after discontinuing treatment, the suppressive activities of EPIT-induced Treg cells were still present at eight weeks after the end of treatment, suggesting that EPIT may induce a more long-lasting tolerance by inducing $CD44^{lo}CD62L^+$ naive cells. Another study monitored the changes in DNA methylation levels during the treatment of peanut-allergic mice with EPIT or OIT (142). Significant hypomethylation of the *FOXP3* promoter in mice was only observed in the $CD62L^+$ Treg cells in the EPIT treatment group, which further verified the potential role of $CD62L^+$ Treg cells in EPIT.

Except for $Foxp3^+$ Treg cells, $Foxp3^-$ Treg cells have also been confirmed to play a role in EPIT. Tordesillas et al. (145) show for the first time that the EPIT treatment with Viaskin® EDS protected OVA-sensitized mice from anaphylaxis and supported the selective expansion of a population of unique gut-homing latency-associated peptide $(LAP)^+$ Treg cells which can directly suppress mast cell activation and lead to sustained clinical protection. It was confirmed by using Viaskin® EDS equipped with OVA-Alexa Fluor 647 in another research (146). Moreover, a high expression level of panmucosal homing marker CCR6 and gut-homing marker CCR9 were observed on the surface of these $Foxp3^-LAP^+$ Treg cells, showing that there was unique imprinting of gut-homing capacity on this Treg-cell subset (145). Furthermore, Dioszeghy et al. (147) found that EPIT treatment effectively induced the production of both $CD25^+Foxp3^+CD62L^+$ Treg cells and $Foxp3^-LAP^+$ Treg cells in the LNs and spleen of mice. They also measured the Treg cell subtypes in the spleen and LNs after eight weeks of EPIT treatment and found that $CD25^+Foxp^+CD62L^+$ Treg cells were still increased compared to two weeks of treatment, but no $Foxp3^-LAP^+$ Treg cells were observed (147). Dioszeghy's research showed that the production of $Foxp3^-LAP^+$ Treg cells is temporary, and the author speculated that $Foxp3^-LAP^+$ Treg cells might participate in the first mechanistic steps of EPIT to induce $CD25^+Foxp3^+$ Tregs (147). Besides, we speculate that this may be related to the local effects of LAP^+ Treg cells, and future studies should further examine the number and proportion of LAP^+ Treg cells in the intestine.

The above research results indicate that EPIT may be a potentially safe, effective, and non-specific treatment for food allergies, which can induce Treg cells of a specific phenotype and immune tolerance. While EPIT could focus on the treatment of some severe food allergens, how the antigen induces the production of Treg cells remains unclear. Dioszeghy et al. (101) used flow cytometry to analyze the phenotype of immune cells in the skin and LNs after using viaskin® EDS loaded with OVA on intact skin for different times. They found that when applied viaskin® EDS on intact skin, the allergen is specifically captured by APCs, especially for DCs, and DCs would further migrate through the dermis to the LNs. Tordesillas et al. (146) applied Viaskin® EDS loaded with OVA to mice's skin to determine how antigen applied topically to healthy skin is acquired and presented by skin DC subsets to generate LAP^+ Tregs. The results showed that $CD11b^+$ $CD64^+$ macrophages acquired most of the antigen reaching the dermis, and the OVA^+ $CD11c^+$ $MHCII^+$ population in the dermis was predominantly $CD11b^+$ cDC2 phenotype. However, only LCs and cDC2s were the main subtypes that presented antigens in the epidermis to the dLNs. Through further cell co-culture experiments (LC or cDC2s co-cultured with DO11.10 mouse $CD4^+$ T cells) and animal experiments with anti-CSF1R (deplete LCs) or langerin-DTR mice or *CD11c-Cre x IRF4$^{fl/f}$* mice, it was

found that cDC2s, rather than LCs, are sufficient for the presentation of topical antigen to $CD4^+$ T cells *in vivo*. Moreover, through co-culture with DO11.10 $CD4^+$ T cells, they found that only $PDL2^+$ cDC2s were able to induce proliferation of responder T cells and mainly promoted the production of $LAP^+CCR4^+CCR6^{low}$ Treg cells. Dioszeghy et al. (147) also used Viaskin® EDS loaded with OVA to study the mechanism by which EPIT treatment induces Treg cells and immune tolerance in an OVA-sensitized mouse model. The phenotypes of APCs and Treg cells in the skin and skin-draining LNs (sdLNs) were analyzed by flow cytometry. In agreement with Tordesillas et al. (146) findings, they found that the allergens in the skin of sensitized mice were taken up by LCs and cDC2s during EPIT treatment and migrated to the sdLNs to induce the production of both $CD4^+CD25^+$ $Foxp3^+CD62L^+$ Treg cells and $Foxp3^-LAP^+$Treg cells. However, Dioszeghy et al. (147) found that LCs depletion significantly reduced the migration of OVA^+CD11^+ cDC2s to sdLNs, and weakened allergens' absorption and the induction of $Foxp3^+$ Treg cells, especially $Foxp3^+CD62L^+$ Treg cells. These changes ultimately led to a failure to induce desensitization and sustained unresponsiveness (SU). The two research methods (146, 147) are basically similar, but Tordesillas et al. (146) did not pre-sensitize mice, so we speculate that the sensitization state of mice plays a vital role in the role of LCs in EPIT treatment. In Yu et al.'s research (136), they used intravital confocal imaging and flow cytometry to analyze the antigen uptake process after PDL-MNA loaded with OVA administration. The results showed that APCs' main phenotypes that took up and processed the antigen in the skin were $CD11b^+CD11c^-F4/80^+$ macrophage cells and $CD11b^+CD11c^+F4/80^+$ macrophage-like cells, which is consistent with the findings of Tordesillas et al. (146). However, they did not find that DCs or LCs played a unique role in the antigen uptake process or conduct further analysis on the cells that play a role in antigen migration. In view of the difference between Viaskin® EDS and PDL-MNA, we speculate that the integrity of the skin barrier may have an impact on the antigen presentation during EPIT treatment. In addition, they compared PLD-MNA with powdered allergens (EPIT) and liquid allergens (SCIT) for treatment. They found that powdered allergens are superior to liquids in attracting immune-regulatory macrophages and inducing immune tolerance in sensitive animals. Moreover, Tordesillas et al. (146) also tested antigen presentation and immune tolerance induction in hairless SKH1 mice with abnormal hair follicle development during EPIT treatment. They found that in SKH1 mice, the delivery of antigen to sdLNs was almost completely abolished, indicating that the integrity of hair follicles is also essential in the antigen presentation process of EPIT. More interestingly, this phenomenon is consistent with the observation that Treg cells in human skin are preferentially located in hair follicles, and that skin with high hair density has a higher proportion of Treg cells than skin with low hair density (148). Besides, Rodriguez et al. (149) also found that about 20% of $CD4^+$ T cells in normal adult skin are Treg cells expressing specific surface molecules, and most of them have an activated effect memory phenotype, which provides conditions for EPIT to induce immune tolerance.

In summary, we can know that in EPIT, the antigens acting on the skin surface can be captured and processed by macrophages, DCs, and LCs, and then further presented by DCs and LCs in the LNs to naïve $CD4^+$ T cells, thereby inducing immune tolerance. Nevertheless, which signaling pathways or signaling molecules involved are still being further explored. Dioszeghy et al. (144) found that the surface of $CD4^+CD25^+Foxp3^+$Treg cells induced by EPIT expressed chemotactic cytokine receptors such as CXCR3 (Th1), CCR4 (Th2), CCR8 (Th2), CCR6 (Th17), CCR9 (gut), and CLA (skin), and the expression levels of CCR6, CCR8, CCR9, and CLA were maintained after the end of immunotherapy, suggesting the induction of a more long-lasting tolerance. Interestingly, only EPIT-induced $CD4^+CD25^+Foxp3^+$Treg cells expressing CLA also expressed CCR9 (**Figure 2**), while OIT-induced Tregs expressed CCR9 but not CLA (144). The above results indicated that these $CD4^+CD25^+Foxp3^+$ Treg cells induced through the skin have obtained intestinal homing properties, while in OIT treatment, Treg cells are locally induced in the mesenteric lymph nodes (mLNs) and only have gut homing properties. This may also be one of the reasons why EPIT rather than OIT can induce systemic immune tolerance. Tordesillas et al. (145) also showed that the use of EPIT to treat mice could produce specific LAP^+Foxp3^- Treg subgroups that highly expressed CCR9 and CCR6. These Treg cells do not function by inhibiting IgE antibodies but directly inhibit mast cells' activation, leading to sustained protection against food-induced allergic reactions. Furthermore, Dioszeghy et al. (144) also showed that EPIT-induced Treg cells are CTLA-4-mediated, rather than IL-10-dependent. More specifically, they used the *in vitro* restimulation of splenocytes in the presence of anti-IL-10 or anti-CTLA4 blocking antibodies to analyze the mechanisms of suppression by $CD4^+CD25^+Foxp3^+$ Tregs cells. The suppression of Th2 cytokine production with EPIT was utterly blocked by anti-CTLA4 rather than anti-IL-10, indicating that the effect of EPIT on $CD4^+CD25^+Foxp3^+$Treg cells is a CTLA-mediated action. Although IL-10 may not be involved in the induction of Treg cells, it still plays an essential role in EPIT treatment. In Yu et al.'s study (81), they found that the macrophage-like cells that produced TGF-β and IL-10 were significantly higher in the EPIT group treated with PLD-MNA than the SCIT group or the sham group. Moreover, the high levels of TGF-β and IL-10 in the skin of PLD-MNA-EPIT-treated mice seem to be consistent with the increased level of $CD4^+CD25^+$ Treg-like cells in the spleen, which indicates that IL-10 and TGF-β may play a role in EPIT treatment. However, its specific mechanism of action still needs to be further explored. Moreover, Mondoulet et al. (142) found that EPIT can lead to a unique and stable epigenetic signature in specific T cells, namely the Th2 cell *Gata3* promoter hypermethylation and $Foxp3^+CD62L^+$ Treg cell *Foxp3* promoter hypomethylation. This specific epigenetic signature is compartments with the down-regulating key Th2 regulators and up-regulating Treg transcription factors, which may explain the sustainability of protection and the observed bystander effect.

As far as current research is concerned, the mechanism of EPIT for food allergies can be summarized as follows (**Figure 2**). First, Antigen applied on the skin surface can be captured,

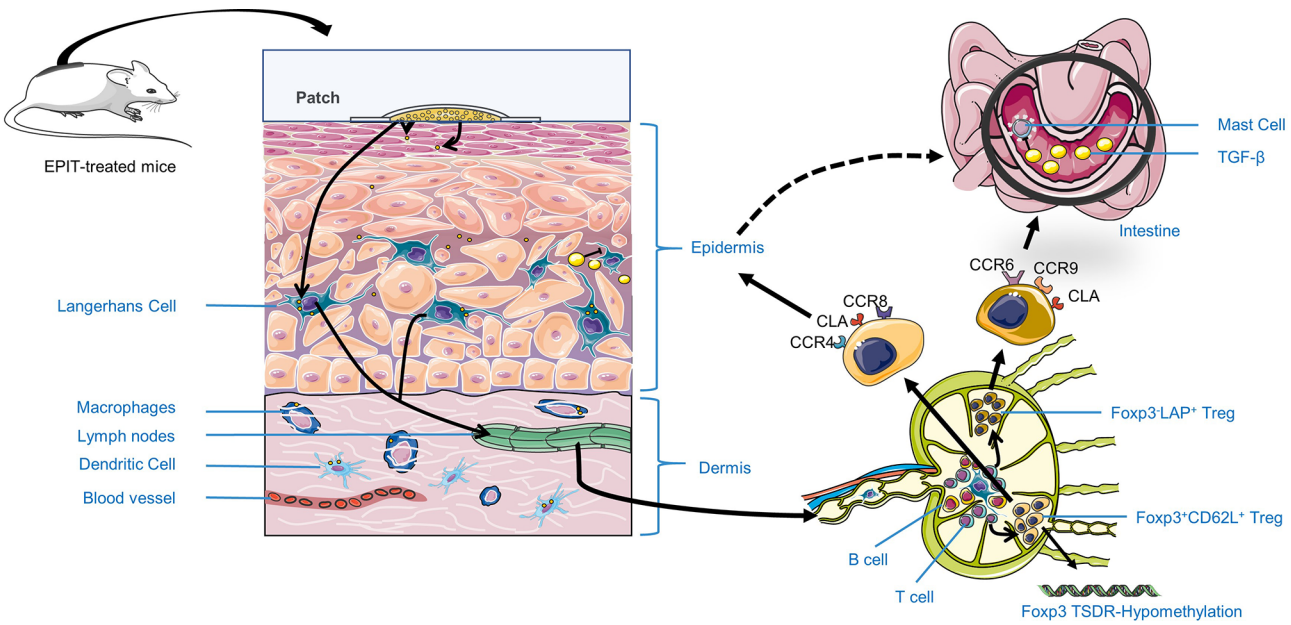

FIGURE 2 | The possible mechanism of EPIT treatment of food allergy to induce immune tolerance. First, Antigen applied on the skin surface can be captured, processed, and presented in the LNs by Antigen-presenting cells (APCs), such as LCs in the epidermis, and macrophages, DCs in the dermis. Besides, they can promote more APCs aggregation by secreting TGF-β or IL-10. Second, APCs migrate to lymph nodes and promote naive T cells distinguish into Treg cells by secreting TGF-β, reducing Foxp3 TSDR methylation level or direct contact. Most importantly, EPIT will exert its immune tolerance by inducing $CD4^+CD25^+Foxp3^+$ Treg cells of a specific phenotype, especially for Foxp3+$CD62L^+$ Treg. Besides, $Foxp3^-LAP^+$ Treg may exert local effects by expressing intestinal homing molecules CCR6, CCR9, and skin-homing molecules CLA, CCR4, related to the higher safety EPIT treatment.

processed, and presented in the LNs by Antigen-presenting cells (APCs), such as LCs in the epidermis, and macrophages, DCs in the dermis. Besides, they can promote more APCs aggregation by secreting TGF-β or IL-10. Second, LCs and cDC2s migrate to the LNs, and cDC2s promote T cells by secreting TGF-β, directly contacting (as described in the regulatory T cell section), reducing *Foxp3* TSDR methylation levels or upregulating GATA3 methylation levels (142) to down-regulate Th2-type immune response. In addition, EPIT exerts its immune tolerance by inducing Treg cells of a specific phenotype, especially $CD4^+CD25^+Foxp3^+CD62L^+$ Treg cells, which play an essential role in the induction of immune tolerance. Simultaneously, $Foxp3^-LAP^+$ Treg cells may be involved in the generation of $CD4^+CD25^+Foxp3^+$ Treg cells and act locally by expressing chemotactic cytokine receptors as CCR6 and CCR9, which are related to the induction of systemic immune tolerance in EPIT treatment (**Figure 2**). Finally, EPIT-induced Treg cells may inhibit the effects of B cells, mast cells, and eosinophils through the direct mechanism (1–3), and (7) described in the regulatory T cells section. This hypothesis is supported by EPIT's observed effectiveness in these allergic mice [the reduction of clinical symptom scores, decrease in allergen-specific IgE (sIgE) levels, increase in IgG levels, and inhibition of mast cells and basophils' infiltration]. The specific mechanism of action of EPIT-induced Treg cells to induce immune tolerance remains to be further confirmed.

PERSPECTIVES

The current mouse model provides essential insights into the EPIT mechanism. However, the mechanism of immune tolerance induced by EPIT has not yet been fully explained. First, we need to determine the specific types of APCs that play a role in EPIT therapy. For example, Dioszeghy et al. (147) reported that LCs are required to induce Treg cells. In contrast, Tordesillas et al. (146) suggest that LCs are redundant and $CD11b^+$ cDC2s are sufficient to present topical antigen to $CD4^+$ T cells *in vivo*. Whether the sensitization status of mice or other mechanisms plays a crucial role in this difference remains explored. Besides, the effects of other APCs, including macrophages, need to be further studied. In addition to specific APCs, Toshiyuki et al. (150) confirmed the contribution of Notch signaling to the establishment of sustained unresponsiveness to food allergens by OIT. The cytokines and signaling pathways involved in the process of antigen presentation by EPIT also required further study. Once these processes are transparent, adjuvants could be used to target APCs and other molecules to assist AIT treatment in promoting the efficacy or reducing the side effects of the treatment. For example, Korotchenko et al. (151) used carbohydrates coupled with allergens to target and stimulate DCs. It shows that the IgE-binding ability of the new glycoconjugate could be reduced, and the side effects of EPIT treatment were significantly reduced. Second, it is necessary to explore further the type, phenotype,

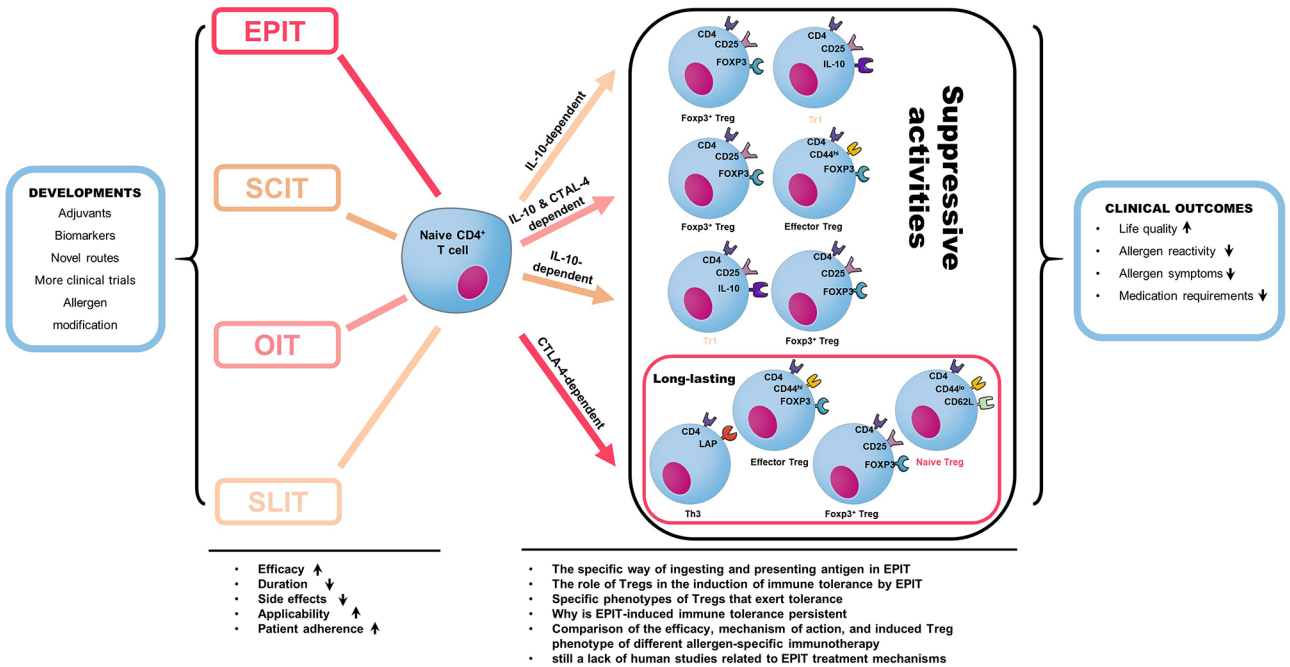

FIGURE 3 | Different allergen-specific immunotherapy can induce the production of different regulatory T cell subtypes. $CD4^+CD25^+Foxp3^+$ Tregs were induced by the three treatment routes but with more significant numbers induced by EPIT. This difference is due to an increase in naive Tregs in EPIT because the induction of effector Tregs was similar in EPIT, OIT, and SLIT, and only EPIT induced naive Tregs. EPIT and OIT also increased the level of $CD4^+LAP^+$ cells (Th3), whereas SLIT induced IL-10^+ Tr1 cells. The suppressive activity of EPIT-induced Tregs did not depend on IL-10 but required CTLA-4, whereas OIT acted through both mechanisms, and SLIT was strictly dependent on IL-10. Furthermore, whereas OIT- or SLIT-induced Tregs lost their suppressive activities after treatment was discontinued, the suppressive activities of EPIT-induced Tregs were still effective eight weeks after the end of treatment, suggesting the induction of a more long-lasting tolerance. Moreover, Allergen-specific immunotherapy is the only option for the long-term cure of allergic diseases. It has been used for food allergy treatment research in recent years. The latest developments in the specific field of allergy and immunology aim to improve the efficacy, applicability, and patient compliance with treatment while reducing side effects and duration. Novel administration routes, the definition of biomarkers for better monitorization of therapy success, novel adjuvants for increased AIT efficacy, and the development of allergen preparations with increased antigenicity and decreased allergenicity allergoids all serve these crucial goals. The final result of AIT is called allergen-specific tolerance, which is a state of active immune response due to changes in immune cell function. A successful AIT's clinical outcome can reduce drug demand, allergen reactivity, and allergic symptoms and improve life quality.

and function of Treg cells produced by EPIT, such as $Foxp3^-LAP^+$ Treg cells and $CD4^+CD25^+$ $Foxp3^+CD62L^+$ Treg cells. Determining which type of cells exerts immune tolerance will provide new insights for Treg cells as a new type of immunotherapy target to treat food allergies. Third, it is necessary to explain how EPIT therapy induces systemic immune tolerance, including how it alleviates skin, digestive, and respiratory allergies and, more importantly, clarifies the role of Treg cells. Fourth, we should also compare Treg cells' role in different allergen-specific treatments, especially the phenotype, function, and persistence of the Treg cells produced by different methods. Finally, there is still a lack of human studies related to EPIT treatment mechanisms, which can provide an essential theoretical basis for EPIT treatment effectiveness and future practical applications (**Figure 3**).

A better understanding of allergen tolerance's underlying mechanisms and the roles and interactions of cells will support developing a more suitable, easily administered, durable, effective, safe, and patient-friendly treatment. EPIT, as a potential treatment for food allergies, has been shown to have high safety and specific therapeutic effects. Because the efficacy of EPIT is still limited, elucidating its mechanism of action and improving its efficacy is still the focus of current research. Improving the allergens used in EPIT treatment, and the development of adjuvants are potential research directions to enhance the efficacy of EPIT. EPIT may be a genuinely effective new method for treating food allergies, but it is clear that our work is not yet done, and the best treatment protocol and mechanisms need to be elucidated.

AUTHOR CONTRIBUTIONS

GL mainly completed the writing and sorting of the article. ML, JW, and YM provided some ideas and insights. HC revised the article. All authors contributed to the article and approved the submitted version.

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Antigen-Specific Treg Therapy in Type 1 Diabetes – Challenges and Opportunities

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Regulatory T cells (Tregs) are key mediators of peripheral self-tolerance and alterations in their frequencies, stability, and function have been linked to autoimmunity. The antigen-specific induction of Tregs is a long-envisioned goal for the treatment of autoimmune diseases given reduced side effects compared to general immunosuppressive therapies. However, the translation of antigen-specific Treg inducing therapies for the treatment or prevention of autoimmune diseases into the clinic remains challenging. In this mini review, we will discuss promising results for antigen-specific Treg therapies in allergy and specific challenges for such therapies in autoimmune diseases, with a focus on type 1 diabetes (T1D). We will furthermore discuss opportunities for antigen-specific Treg therapies in T1D, including combinatorial strategies and tissue-specific Treg targeting. Specifically, we will highlight recent advances in miRNA-targeting as a means to foster Tregs in autoimmunity. Additionally, we will discuss advances and perspectives of computational strategies for the detailed analysis of tissue-specific Tregs on the single-cell level.

Keywords: antigen-specific Treg therapy, autoimmunity, T1D, microRNAs, tissue Tregs, single-cell multi-omics integration, TCR specificity prediction

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INTRODUCTION

The body's immune system has evolved to effectively defeat and destroy infiltrating foreign pathogens. In order to prevent autoimmune reactions directed against the body's own cells, our immune system employs sophisticated mechanisms of self-tolerance. On the T cell level, self-tolerance is executed in the thymus by deletion of T cells with self-reactive TCRs (central tolerance). Outside of the thymus, peripheral tolerance is maintained by specialized cells, including so-called regulatory T cells (Tregs). Tregs are characterized by the high expression of the interleukin-2-receptor-aplpha chain (CD25) and the transcription factor Foxp3, which is the master regulator of Tregs phenotype and function (1–4). The critical importance of Tregs for the maintenance of self-

tolerance is illustrated by severe multi-organ autoimmunity in humans with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (5) and mice with Scurfy mutations (6), both resulting from mutations in the *Foxp3* gene. Tregs develop in the thymus, referring to thymic Tregs (tTregs), and harbor a TCR repertoire that is skewed towards self-antigens. Additionally, Tregs can likewise be induced in the periphery in an antigen-specific manner, so called peripheral Tregs (pTregs), with a TCR repertoire different from their tTreg counterparts (7). Considerable research has been conducted in order to induce disease-relevant antigen-specific Tregs with the goal to restore mechanisms of tolerance and interfere with unwanted immune reactions in allergies and autoimmunity. Accordingly, we and others have shown that Treg induction requires stimulation *via* the TCR and it has become apparent that fine-tuned TCR signals are needed to efficiently induce Tregs (8–11). Here, we will discuss promising results for antigen-specific Treg therapies in allergy and specific challenges for such therapies in autoimmune diseases, with a focus on type 1 diabetes (T1D) as well as opportunities for antigen-specific Treg therapies in T1D.

ADVANCES IN ANTIGEN-SPECIFIC TREG THERAPIES IN ALLERGY

Antigen-specific therapy is a long-envisioned goal for the treatment or prevention of autoimmune diseases. The ability of Tregs to regulate immune responses not only *via* direct inhibition of effector T cells with the same specificity but also *via* modulation of antigen-presenting cell (APCs), a process called bystander suppression, makes Tregs an important target for tolerizing therapies (12). Currently, approaches based either on the expansion, manipulation and transfer of autologous Tregs as well as the *in vivo* induction with antigen are extensively studied. While the *ex vivo* expansion of polyclonal Tregs has proven to be safe in the clinic the efficacy is largely dependent on disease-relevant antigen-specific Tregs. However, their very low frequency in the case of autoimmune diseases necessitates the manipulation of Tregs before transfer [reviewed in (13)]. This includes the forced expression of FOXP3 in autoantigen-specific effector T cells as well as the expression of disease relevant TCRs on isolated Tregs [reviewed in (13)]. Although results from preclinical studies are promising, the long-term fate of these engineered Tregs is not fully understood and especially the differentiation into pro-inflammatory lineages might be a safety concern. The alternative of induction of Tregs with antigen administered directly to the patients is more cost-effective and its safety has been demonstrated in a variety of clinical trials. Even though clinical translation of such tolerizing therapies has been challenging, several examples relying on different forms of antigen-delivery and tolerization protocols from pre-clinical and clinical trials highlight the potential of such strategies.

Desensitization to allergens is a common practice for the treatment of severe allergies. However, only a few studies have addressed the effect of such antigen-specific desensitization protocols on Tregs. Importantly, oral immunotherapy with peanut proteins in allergic patients led to an increase in peanut protein-specific $FOXP3^+$ Tregs within peripheral blood mononuclear cells (PBMCs) 6 and 12 months after the treatment started (14). Interestingly, in a follow-up study focusing more specifically on Tregs, it became evident that the increased frequencies of peanut-protein specific Tregs were associated with enhanced DNA demethylation of the *FOXP3* locus (15), a measure for maintenance of FOXP3 expression and therefore for the stability of the Treg phenotype (16). These findings highlight that antigen-specific therapy can not only enhance Treg frequencies but also positively affect Treg characteristics including their stability.

CHALLENGES FOR ANTIGEN-SPECIFIC TREG THERAPY IN AUTOIMMUNITY AND T1D

Autoimmune diseases like T1D affect millions of people worldwide with a steadily rising incidence. Currently, curative treatments for autoimmune diseases do not exist and available therapies rely on the treatment of symptoms often involving immunosuppressive reagents that can have severe side effects. The antigen-specific induction of disease-relevant Tregs offers the opportunity to restore natural tolerance mechanisms in the absence of immune side effects induced by general immune suppression and is therefore a long-standing goal for the treatment or prevention of autoimmune diseases. We were able to demonstrate that in the peripheral blood of children at risk to develop T1D, insulin-specific Treg frequencies are reduced during the onset of islet autoimmunity, while higher frequencies are associated with a slow progression to clinically overt T1D (17). These findings directly support the concept of inducing these insulin-specific Tregs to delay the progression to clinically symptomatic disease. However, the translation of antigen-specific Treg therapies for autoimmune diseases into the clinic remains challenging and most studies using oral insulin treatments for tolerization in T1D conducted so far failed to meet their primary outcome (18, 19). Nevertheless, post-hoc analysis revealed a delay in progression in a subset of these treated participants (20). One analytical caveat of clinical trials studying Treg therapies has been the divergence of protocols for Treg identification in peripheral blood. While in the mouse setting Foxp3 is expressed exclusively by Tregs, human effector T cells can transiently express intermediate levels of FOXP3. Accordingly, most researchers characterize human Tregs as $CD25^+CD127^{low}FOXP3^+$. It has become apparent though, that even those more stringently defined Tregs are heterogeneous in their composition. Not only can Tregs co-express classical effector T cell transcription factors (e.g. TBET, RORC, GATA3) which affects their migration and function, but they also vary in their activation state and functionality. This is especially evident in the divergent expression of CD45RA, with $CD45RA^-$ Tregs being antigen-experienced and having a higher

suppressive activity [reviewed in (7)]. According to this heterogeneity, divergent markers have been used for the identification of Tregs in clinical trials which contributes to the difficulties in assessing translatability. Importantly, researchers are starting to analyze antigen-specific immune responses in such clinical trials in more mechanistic detail, which will help to define critical parameters, such as the optimal dosing of oral insulin. Additionally, other factors need to be critically considered, including the route of administration and the chosen antigen but also the time point of administration within the disease course.

We know from murine studies that the efficient *de novo* induction of Tregs from naïve T cells *in vivo* requires the stimulation with a strong-agonistic ligand for the TCR supplied under subimmunogenic conditions (8, 9). Higher immunogenic doses of antigen on the other hand activate the Pi3k-Akt-mTOR pathway, thereby directly inhibiting Treg induction (10). We used immunodeficient HLA-DQ8-transgenic NOD-Scid-IL2Rγ knockout (NSG) mice reconstituted with human hematopoietic stem cells to study requirements for human Treg induction *in vivo*. Importantly, these humanized mice develop a functional human immune system, including the positive selection of autoreactive insulin-specific $CD4^+$ T cells in the thymus (17, 21). Using this system under steady state conditions in the absence of autoimmune activation, we were able to demonstrate that, similar to the murine setting, subimmunogenic doses of strong-agonistic insulin variants are able to induce human Tregs *in vivo* (17).

In contrast to the steady state, we demonstrated that during the onset of islet autoimmunity the capacity to induce Tregs from naïve T cells from peripheral blood is significantly impaired (22). Importantly, this impairment in Treg induction was not limited to the insulin-specific population, but was likewise observed for hemagglutinin-specific and polyclonal Treg induction, highlighting a broad defect in Treg induction (22). Furthermore, we were able to show that a reduction in the activation threshold of insulin-specific T cells during the onset of islet autoimmunity limits the possibility of subimmunogenic stimulation for efficient Treg induction (22). Apart from defects in Treg induction during islet autoimmunity, we likewise observed reduced Treg stability as indicated by increased DNA methylation of the conserved non-coding sequence 2 (CNS2) of the *Foxp3* locus both in non-obese diabetic mice (NOD, mouse model for T1D) with islet autoimmunity as well as in children with overt T1D (23). The *Foxp3* CNS2 is completely demethylated in stable Tregs, while its methylation leads to the loss of Foxp3 expression and the Treg phenotype (16). Importantly, this defect in Treg stability in NOD mice was observed already at a young age, shortly after weaning, indicating a possible causative role in disease development and progression as opposed to a mere consequence of the ongoing autoimmune process (23). The identified impairments in Treg induction and stability directly highlight the importance of considering the time point of administration of antigen-specific Treg inducing therapies. Our *in vitro* and *ex vivo* data suggest limitations in the efficacy of such treatments during the first years after development of islet autoimmunity. In addition, these findings strengthen the rationale of considering preventive strategies in genetically at-risk patients, before the onset of overt islet autoimmunity, for future antigen-specific Treg targeting in man. Accordingly, for T1D pilot results from the Pre-POINT study, the first study to administer daily oral insulin to children at risk to develop T1D, but before the start of the autoimmune reaction, resulted in enhanced frequencies of insulin-specific $CD4^+$ T cells with regulatory features (24). These preliminary results are currently further investigated in the larger POINT study for efficacy (25).

OPPORTUNITIES FOR ANTIGEN-SPECIFIC TREG THERAPY IN T1D

The finding that Treg induction potential is significantly limited during onset of islet autoimmunity (22) highlights the concept that antigen-specific Treg induction in the presence of ongoing autoimmune activation will benefit from combinatorial immune targeting. Specifically, a combination with treatments that control aberrant immune activation while fostering Tregs will be critical in order to broaden the window of opportunity for Treg induction.

miRNA Targeting to Foster Tregs in Islet Autoimmunity

With the goal to understand mechanisms of impaired Treg induction, we focused on microRNAs (miRNAs). miRNAs are small non-coding RNAs that can sequence-specifically inhibit their target mRNAs. miRNAs usually target a multitude of different mRNAs, thereby regulating entire signaling pathways and complex cellular states, such as T cell activation, which makes them important targets for immunotherapies (26–28). Using miRNA sequencing of $CD4^+$ T cells from peripheral blood of children with or without ongoing islet autoimmunity, we were able to identify several differentially regulated miRNAs and investigated three in more detail. Specifically, we focused on miRNAs that are predicted to target negative regulators of T cell activation and could therefore potentially inhibit Treg induction [reviewed in (29–31)].

We were able to demonstrate that miRNA92a-3p, a member of the miRNA17~92 cluster of miRNAs which was shown to induce lupus-like autoimmunity when overexpressed in mice (32), regulates human T follicular helper (TFH) cell differentiation (33). TFH cells are an integral part of the humoral immune response because of their ability to help B cells produce high-affinity antibodies [reviewed in (34)]. Accordingly, we found $CXCR5^+$ insulin-specific TFH cell frequencies to be increased during onset of islet autoimmunity, which was directly correlated with miRNA92a-3p expression. Importantly, miRNA92a-3p targets negative regulators of T cell activation (e.g., PTEN, PHLPP2, FOXO1, CTLA4) and thereby simultaneously reduces Treg induction. Hence, inhibition of miRNA92a-3p enhanced while a miRNA92a-3p mimic reduced Treg induction (33).

Furthermore, we investigated miRNA181a-5p, which has been demonstrated previously to regulate the signal strength of the TCR stimulus in developing T cells in the thymus (35). In line with excessive T cell activation observed during recent onset of islet autoimmunity, we found miRNA181a-5p to be specifically increased in $CD4^+$ T cells from peripheral blood of children with recent activation of islet autoimmunity. Importantly, we found that higher expression of miRNA181a-5p enhances the expression of Nfat5 involving mechanisms of increased TCR- and co-stimulation and that enhanced Nfat5 expression negatively affects Treg induction. Accordingly, inhibiting either miRNA181a-5p or Nfat5 augmented *in vitro* Treg induction, while inhibiting miRNA181a-5p in Nfat5 deficient T cells had no effect on Treg induction. These findings thereby highlight, that miRNA181a-5p mediated impairments in Treg induction are dependent on Nfat5 (22).

In a third study we used high throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) to show, that miRNA142-3p directly targets the methylcytosine deoxygenase Tet2. Importantly, TET proteins catalyze the first step of DNA demethylation and can thereby impact the epigenetic landscape (36). We were able to link increased expression of miRNA142-3p and resulting reduced Tet2 expression with impairments both in Treg induction as well as in Treg stability. Accordingly, the inhibition of miRNA142-3p was able to enhance Treg induction and enable induced Tregs to retain their Foxp3 expression to a higher degree than their untreated counterparts (23).

Importantly, the inhibition of all three miRNAs or the downstream molecule Nfat5 directly *in vivo* in NOD mice with ongoing islet autoimmunity resulted in enhanced frequencies of Tregs accompanied by a reduction in the clinical disease score of the mice (22, 23, 33). These preliminary findings highlight the potential of miRNA-targeting as immunotherapy in T1D. Notably, a miRNA inhibitor is currently being investigated in a clinical trial as treatment for hepatitis C virus infections, thereby indicating the feasibility of miRNA modulation as immunotherapy (37). However, miRNAs are important regulators of cellular functions and can have distinct properties depending on the cell type. Therefore, the use of miRNA modulation as immunotherapy will be largely dependent on the cell type-specific targeting of the therapy. Specifically, the targeted delivery of miRNA inhibitors or mimics to immune cells or even immune cell subsets will greatly improve their use as immunotherapeutics. Here, it will be especially important to identify specific signatures for targeting defined subsets of immune cells, e.g., tissue-specific Tregs in the target organ, the pancreas.

Targeting Tissue-Specific Tregs

Apart from their canonical function of immune suppression, it is now well accepted that Tregs likewise take residence in tissues, where they play important roles in maintaining tissue homeostasis. These tissue Tregs were found to express specific gene signatures that are distinct from their circulating counterparts. Such tissue specific Treg gene signatures have been identified for Tregs from specific tissues, while they have been especially well studied for Tregs in the muscle and adipose tissue [reviewed in (38)]. Importantly, some signature genes are universal for tissue Tregs while others are more unique to Tregs from distinct tissues, e.g., the expression of the transcription factor PPARγ on adipose-tissue residing Tregs (39). Apart from their gene expression signature, TCR sequencing of tissue resident Tregs has identified a distinct TCR repertoire and clonal expansion of certain TCRs, indicating the response to tissue-specific antigens (40). Importantly, treatment with the PPARγ agonist pioglitazone, which is used for the treatment of type 2 diabetes because of its positive effects on metabolic health and local inflammation, was shown to expand adipose tissue Tregs, which supports the idea of targeting tissue-specific Tregs for the treatment of diseases (39).

While Tregs in adipose tissue, muscle and the intestine have been studied extensively, only very little is known about Tregs in the pancreas. A study by the group of Christophe Benoist demonstrated that the diabetic lesions in NOD mice are enriched in $CXCR3^+$ Tregs and that the expression of CXCR3 is dependent on Tbet. More importantly, they showed that the ablation of Tbet in Tregs accelerates the disease and overcomes the usually present sex-bias in NOD mice (41). Interestingly, $Tbet^+$ Tregs were also found in the lamina propria of patients with inflammatory bowel disease (42) as well as in patients with multiple sclerosis (43), where $Tbet^+$ Tregs were shown to contribute to the disease manifestation and being less suppressive (43). Importantly, the reduced suppressive activity was linked to the Ifnγ production of the Tregs which was not elevated in $Tbet^+$ Tregs from the pancreas (41). These findings highlight the possibility of specifically targeting defined Treg subsets within the pancreas for a more tailored immune modulation. However, all studies conducted so far on pancreas residing Tregs focused solely on NOD mice with ongoing insulitis. A more detailed understanding of pancreas residing Tregs and their contribution to immune homeostasis in the steady state will be crucial to advance immune modulation targeted to the pancreas.

As one means to foster advancement in tissue-specific Treg targeting, recent years have seen tremendous progress in the simultaneous analysis of transcriptome, DNA methylation and accessibility, surface protein expression, perturbations, and receptor sequences on the single cell level. In this regard, computational strategies for integration of these complex data sets have enabled an unprecedented description of molecular behavior and identities of individual cells and therefore made it possible to move along to the next level of dissecting tissue Tregs (**Figure 1**).

Defining Tissue-Specific Treg Characteristics Using Single-Cell Multi-Omics Integration

Current single-cell multi-omics methods can measure up to four different omics types at once [reviewed in (44–46)], with the transcriptomics layer often used to connect between the different omics types. These techniques bear high potential for medical

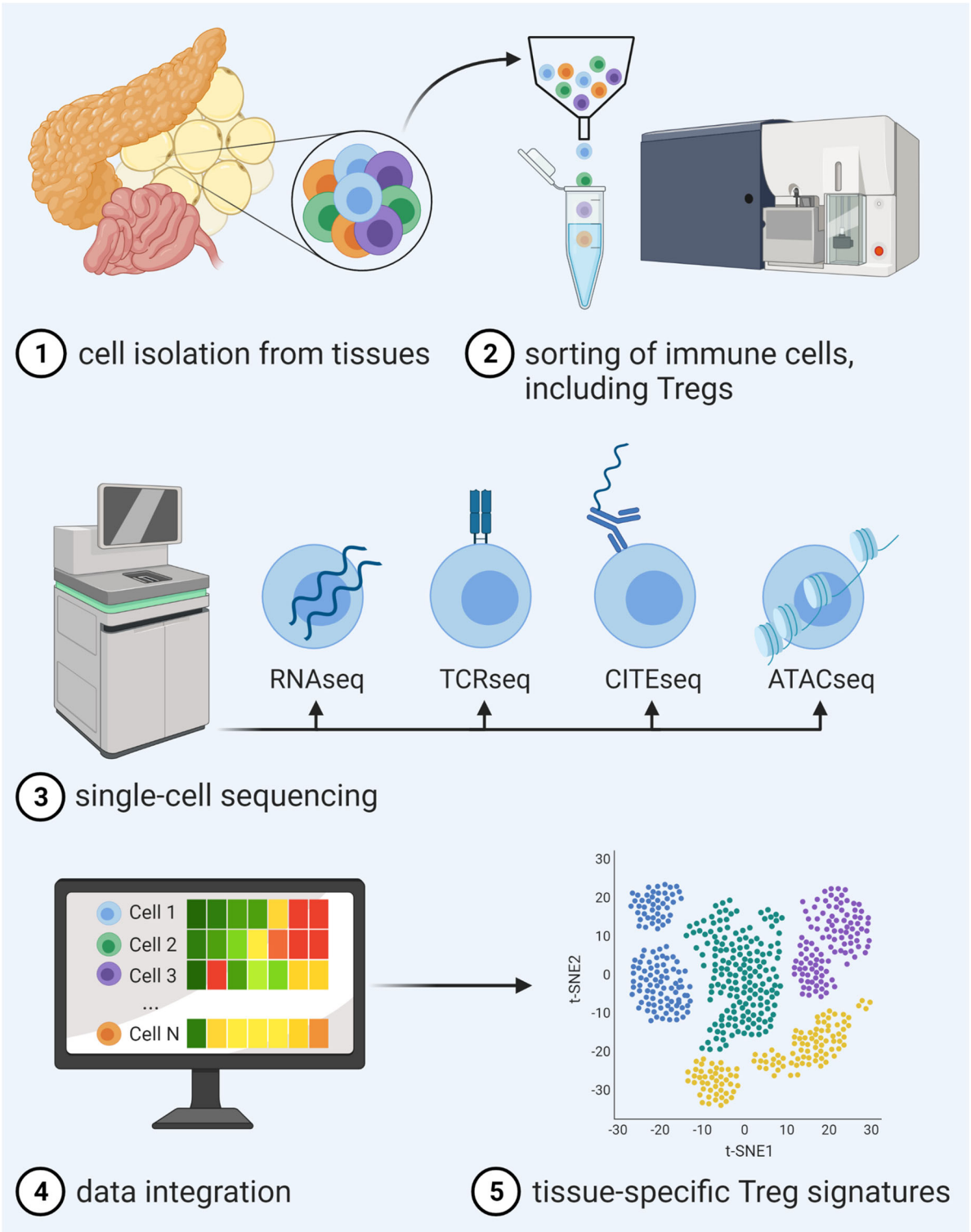

FIGURE 1 | Advancements in single-cell multi-omics integration allow for a detailed analysis of tissue Treg signatures. After isolation and sorting of heterogeneous immune cell populations from tissues and single-cell sequencing of distinct libraries for RNAseq in combination with, e.g., TCRseq, CITEseq or ATACseq, novel computational approaches enable data integration of different traits, thereby enabling unprecedented description of molecular behavior and identities of individual cells within a certain tissue.

research to study individual heterogeneity, drug resistance, or disease progression at an unprecedented level (47, 48). Especially, T cell focused immunological studies will benefit from recent developments as newly arising techniques can also simultaneously reconstruct TCR sequences and determine their specificities for a predefined set of epitopes (49–51). These methods have already greatly advanced our understanding of T cell responses in disease (50, 52–56), and lead to innovative analysis strategies such as the usage of TCR-sequence as natural barcodes to trace the cellular response pre- and post-antigen stimulation *in vivo* (57).

With the rise of single-cell multi-omics approaches, new computational models have been developed that can jointly analyze such multi-modal data [reviewed in (46, 58)]. Several studies used correlation-based approaches to jointly analyze copy number variations (59, 60), DNA methylation (61–63), or protein abundance (64) and gene expression data. Recently, Schattgen et al. proposed an integration approach for TCR and gene expression data based on graph analysis defined on transcriptomic and TCR distances and could uncover known and novel associations between TCR sequences and transcriptomics phenotypes (65). Others used traditional statistical approaches (66), or advanced deep learning methods (67–73) to integrate multiple data sources at once to represent the joint information of all omics-layers. Along these lines, a recent method by Zhang et al. jointly integrated TCR and transcriptomic information using Bayesian clustering based on the TCR sequence and gene expression profile (74). Through this method Zhang et al. could show that joint TCR and gene expression analysis better separates T-cell specificity and captures the antigen binding efficiency gradient better than TCR-information alone (74). Similarly, we introduced a joint TCR-transcriptome deep learning model which additionally captured transcriptional gradients within clonotypes (73). Such methods could be used to further elucidate the relationship between the TCR sequence and transcriptional information of Tregs in autoimmune diseases.

The identification of specific TCRs on tissue Tregs will help to define whether the migration of these cells to the tissue is likely antigen-driven and can also help to facilitate studies on tissue Tregs. In this regard, Diane Mathis group was able to analyze the ontogeny of visceral white adipose tissue (VAT)-residing Tregs by generating a mouse line transgenic for the TCR of an expanded VAT Treg clone (40). Additionally, the transfer of TCR transgenic Tregs has already been tested in preclinical studies for autoimmune diseases (75, 76). These studies mostly rely on the use of effector T cell derived TCRs and it is not entirely clear how that could affect Treg function, migration, and fate after transfer. The identification of tissue- and Treg-specific TCRs in the steady state as well as differences to the disease state might enable us to design such transgenic Tregs more strategically and could therefore help to increase efficacy and safety of TCR transgenic Treg infusions.

However, the identification of TCR sequences is only one side of the coin and a remaining bottleneck for T cell biology is the identification of the peptide-MHC ligands recognized by the identified TCRs. Here, recent advances have been made for experimental identification of epitopes recognized by orphan TCRs in a high-throughput screening of highly complex peptide-encoding oligo pools presented by bar-coded T cell-cytokine capturing APCs (77). Additionally, machine learning has enabled novel computational approaches to predict TCR specificity.

Sequence-based computational methods for TCR specificity analysis can be grouped into two categories: comparison and prediction. TCR comparison approaches impute antigen specificities by either allocating unknown TCRs to T-cell clusters or by assigning pairwise distance scores to TCR sequences with known antigen specificity. When several TCRs specific to the antigens of interest are known, these methods can be used to identify T cells with similar sequences likely to bind to the same antigen. The second category applies machine learning models to directly predict TCR binding to specific epitopes. Since these methods often additionally analyze the epitope sequence, they allow to predict specificity towards previously unknown antigens.

TCR sequences with common epitope specificity carry statistically enriched motifs (78, 79). Methods such as TCRdist (78) and GLIPH (79, 80) compare such common motifs to identify TCR sequences with shared antigen specificities. Other methods were proposed differing in computational approach to match TCRs using sequence similarity (81) or numeric embeddings (82, 83).

While comparison-based methods can serve as a proxy for determining TCR-specificity, such methods fail for novel epitopes without known corresponding TCRs. Machine learning methods can alleviate these issues by learning general rules that guide the T-cell epitope interaction. De Neuter et al. provided a proof of concept by predicting specificity towards one of two B*08 restricted HIV-1 epitopes based on the TCR CDR3β sequence (84). Jurtz et al. additionally incorporated the peptide sequence but observed limited generalization to unknown epitopes (85). Subsequently, different models developed on varying datasets have been proposed with limited improvements (86). In recent years, deep learning methods were introduced (52, 87–89), of which some incorporate additional information such as CDR3α, CDR1 and CDR2 sequences, HLA type, and surface protein counts leading partially to increased prediction performances (52, 86).

These tools will potentially enable the identification of Tregs associated with disease-relevant antigens by predicting the specificity for large libraries of sequenced T cells. By limiting the number of candidates, for which specificity needs to be tested, the time and cost for identifying disease-relevant Tregs will be significantly reduced. However, due to different evaluation methodologies and different datasets, these methods often cannot directly be compared. Therefore, it remains yet to be determined, which model to choose, and to what degree computational tools can be already used for the development of targeted immunotherapies. It is apparent though, that the use of multi-omics techniques for the deep characterization of tissue-specific Tregs can critically contribute to the development and

advancement of Treg-based immunotherapies. TCR transgenic Tregs migrate to the site of immune activation and therefore will facilitate the development of effective and safe therapies. Additionally, identification of surface markers specific to tissue-residing Tregs will enable targeted delivery of therapeutics, e.g., miRNA inhibitors or mimics, to foster Tregs specifically at the site of the autoimmune attack.

CONCLUSION

While advances have been made for antigen-specific Treg inducing therapies e.g. to treat patients with severe peanut allergies, the success of such therapies in autoimmune T1D is still limited. A broad impairment in Treg induction in children during onset of islet autoimmunity highlights the necessity of combinatorial strategies to foster Tregs in order to open the window of opportunity for antigen-specific Treg therapies. miRNA-targeting offers the opportunity to improve Treg induction and stability in T1D. However new strategies to specifically modify miRNAs in specific cell types are needed. Identifying key signatures and characteristics of Tregs residing in the pancreas, the target organ of the disease, will be important to target therapies more specifically to those cells that are directly involved in the disease development and progression. Major advances in the use of single-cell multi-omics integration together with machine learning approaches for TCR specificity prediction have paved the way for a detailed description of individual cells from different tissues and will therefore help to bring antigen-specific Treg therapy to the next level.

AUTHOR CONTRIBUTIONS

IS and FD reviewed the literature and wrote the manuscript. CD and BS reviewed the literature and contributed to the conceptualization of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immune Equilibrium Depends on the Interaction Between Recognition and Presentation Landscapes

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In this review, we described the structure and organization of antigen-recognizing repertoires of B and T cells from the standpoint of modern immunology. We summarized the latest advances in bioinformatics analysis of sequencing data from T and B cell repertoires and also presented contemporary ideas about the mechanisms of clonal diversity formation at different stages of organism development. At the same time, we focused on the importance of the allelic variants of the HLA genes and spectra of presented antigens for the formation of T-cell receptors (TCR) landscapes. The main idea of this review is that immune equilibrium and proper functioning of immunity are highly dependent on the interaction between the recognition and the presentation landscapes of antigens. Certain changes in these landscapes can occur during life, which can affect the protective function of adaptive immunity. We described some mechanisms associated with these changes, for example, the conversion of effector cells into regulatory cells and *vice versa* due to the trans-differentiation or bystander effect, changes in the clonal organization of the general TCR repertoire due to homeostatic proliferation or aging, and the background for the altered presentation of some antigens due to SNP mutations of MHC, or the alteration of the presenting antigens due to post-translational modifications. The authors suggest that such alterations can lead to an increase in the risk of the development of oncological and autoimmune diseases and influence the sensitivity of the organism to different infectious agents.

Keywords: adaptive immunity, immune equilibrium, T-cell receptor repertoire, B-cell receptor repertoire, antigen presentation/recognition, homeostatic proliferation, a rank-size frequency distribution of T- and B-cell receptors, immunopeptidome

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INTRODUCTION

The immune system is a complicated multilevel system of protection from different pathogens that contributes to the multicellularity and maintenance of genetic homeostasis (1–3). The development of adaptive immunity is associated with the appearance of RAG (recombination-activating gene) and two consecutive whole-genome duplications (4) that could be associated with the appearance of vertebrates and a transition from Agnatha to gnathostomes, which occurred around 500 million years ago (5, 6). The most important evolutionary advantage of adaptive immunity seems to be its specificity, which provides high precision and selectivity of the immune system activity. Another

important advantage is the formation of immunological memory, which provides a quick and targeted reaction to the pathogen that the organism faced before (7). The so-called "price" that has to be paid for these advantages is the necessity to re-customize the adaptive immunity and form the immune memory individually in each generation.

The evolutionary development of the adaptive immune response is associated with the appearance of populations of T and B lymphocytes. Their precursors are found at the stage of early vertebrates (8). The main peculiarity of adaptive immunity is the formation of T and B lymphocytes with a high diversity of clones, wherein each clone has a unique antigen-recognizing receptor (TCR—T-cell receptor or BCR—B-cell receptor, respectively). For example, according to the recent data, the clonal diversity of only β chains of TCRs in the peripheral bloodstream is up to 10^8, which does not reflect the whole diversity that is comprised in the different organism compartments because the total number of T cells in the human organism is up to 10^{12} (9). At the same time, a recent evaluation indicates that the potential diversity of $\alpha\beta$ TCRs varies from 10^{20} to 10^{61}, which significantly exceeds the number of unique TCRs in the human organism (10, 11). In the case of B cells, the potential diversity of the BCR repertoire is also great and reaches 10^{20} (12–14). However, as for TCRs, the actual number is lower than the theoretical one and is approximately 10^8–10^9 of unique heavy BCR chains in the peripheral bloodstream (15, 16). These peculiarities lead to high personalization of repertoires, when the major part is private TCRs/BCRs and only a small part can be common in different individuals (public TCRs/BCRs). It is suggested that cross-reactivity plays an important role in the recognition of antigens because the approximate diversity of potential antigens reaches 20^9 and seems to exceed the summed actual diversity of T- and B-cell repertoires (11).

Such diversity is provided due to recombination of V(D)J gene segments of TCRs and BCRs caused by the activation of the RAG gene and due to the effect of terminal deoxynucleotidyl transferase (TdT) at the early stages of lymphocyte maturation (17, 18). The migration of T cells from the thymus begins at the end of the first trimester of the intrauterine development, while TdT, which randomly inserts nucleotides during V(D)J recombination, begins to express only in the middle of the second trimester (19). Thus, the majority of T cells in the fetus to the middle of gestation have zero nucleotide insertion in the region CDR3 (complementarity-determining region-3) (20). Still, their repertoire has a quite high diversity of TCRs due to V(D)J recombination. Unlike TCRs, the diversification of the BCR repertoire occurs earlier. For this reason, at the beginning of the second trimester, the BCR repertoire is characterized by a relatively high diversity, which gradually increases to the time of birth. After the birth, the diversity of the T-cell repertoire continues to increase to the involution of the thymus, while an increase in the diversity of the B-cell repertoire seems to be limited by the age-related degeneration of the bone marrow (20, 21). At the same time, at the early stages of development (less than 14 weeks), both repertoires of T and B cells are characterized by the oligoclonal organization that is replaced with polyclonal one by the 17th week of gestation, which is associated with a progressive increase in the number of sjTRECs (signal-joint T-cell receptor excision circles) and sjKRECs (signal-joint kappa-deleting recombination excision circles) (20). In early childhood, the diversity of T- and B-cell repertoires tends to its maximum (20, 22). Thus, in the fetal period and early childhood, the main diversity of the TCR and BCR repertoires is established that form the general landscape of recognition of antigens, which normally changes insignificantly within the life and tends to decrease with aging (22–24).

It is worth noting that MHC (major histocompatibility complex) molecules influence the formation of TCR repertoires of $CD4^+$ and $CD8^+$ lymphocytes. In other words, allele variants of MHCs limit the diversity of the represented antigens, which in turn, influences the formation of naïve and antigen-experienced TCR repertoires (25–27). At the same time, there are some differences in the formation of TCR repertoires of $CD4^+$ and $CD8^+$. This could be associated with different events observed in the thymus that determine the choice between the $CD4^+$ or $CD8^+$ cell differentiation. Double-positive $CD4^+CD8^+$ lymphocytes that receive a strong TCR-MHC-II signal, quickly stop the expression of CD8 and become single-positive $CD4^+$ lymphocytes. In turn, $CD4^+CD8^+$ lymphocytes that do not receive a relatively strong TCR-MHC-II signal for a long time stop the expression of $CD4^+$ and become single-positive $CD8^+$ lymphocytes (28). Thus, $CD8^+$ lymphocytes undergo a stricter selection in the thymus. Along with the possibility of recognizing epitopes presented by MHC-I, they lose the capability to recognize epitopes presented by MHC-II, which plays an important role in forming the naïve $CD8^+$ TCR repertoire (29). This is confirmed by a small total amount of common TCRβ sequences in the populations of $CD4^+$ and $CD8^+$ lymphocytes, *i.e.* TCR repertoires of $CD4^+$ and $CD8^+$ overlap weakly, and there is a small amount of TCRs capable of reacting with both classes of MHC-I and MHC-II (30). Thus, the specificity of TCRs at the stage of $CD4^+CD8^+$ cells regulates the choice of CD4/CD8 differentiation. Further changes in the $CD8^+$ repertoires could be associated with the allele variants of MHC-I, in particular, with their variants of fastidious or promiscuous binding that initiate oligoclonal or polyclonal variants of the immune response, respectively, by changing the number of certain clones (31–33). The difference between the $CD4^+$ and $CD8^+$ repertoires is in the formation of T-regulatory cells (Tregs) with a relatively high affinity of TCRs to self-antigens at the double-positive stage (34). Thus, the $CD4^+$ repertoire contains cells with relatively high affinity to self-antigens, which is not observed in the $CD8^+$ repertoire.

The formation of the naïve BCR repertoire of B cells is not so dependent on innate immunity. Similar to T cells, during maturation, B cells go through several stages of positive and negative selection. Each B cell can go through several cycles of rearrangement of V genes at different stages of maturation to increase the possibility of the formation of BCRs with the minimal capacity of reacting to self-antigens for the population of B-2 cells and a relatively higher affinity to self-antigens for the

population of B-1a and B-1b cells (35, 36). Further formation of antigen-experienced BCR repertoire occurs in the process of somatic hypermutations (SHMs) during the maturation and activation of B cells on the periphery and under the mediated effect of Th cells, which suggests indirect involvement of the innate immunity (36).

Although the formation of the diversity of Ag-recognizing receptors occurs in a stochastic manner due to random V(D)J recombination and non-template nucleotide insertions (NIs), it is limited by a set of allele variants of MHC genes for a certain subject, at least, for $CD4^+$ and $CD8^+$ cells. Probably, this determines the individuality of the immune response and peculiarity of the homeostasis of the immune system in general in a certain organism. In this review, the authors will shortly describe the main peculiarities that can occur throughout life and affect the immune equilibrium increasing the risk of pathology.

LANDSCAPE OF RECOGNITION

As it was mentioned before, the general landscape of recognition is understood as a diversity of specific antigen-recognizing receptors that include TCRs of $CD4^+$ and $CD8^+$ cells, as well as BCRs of B cells. It is assumed that the higher the diversity of antigen-recognizing receptors, the wider the range of antigens that can potentially be recognized by the immune system, and, thus, the higher the effectiveness of the immune response against pathogens and altered self-antigens (37). It is suggested that the maintenance of auto-tolerance and the efficiency of anti-cancer immunity are also associated with the diversity of antigen-recognizing receptors of Tregs, which is critical in the context of antigen-specific action of these cells (38). Since the number of cells in the organism is limited, the formation of protective diversity should be based on the relation between the general diversity and the size of each antigen-specific clone. This idea is described in the Protecton Theory (39), wherein the protecton is the minimum number of cells of certain antigen specificity required for the timely provision of the sufficient number of effector cells per the unit of the body volume for the efficient protection against antigens (10, 39). Thus, knowledge of the clonal organization of T- and B-cell repertoires is important for the understanding of consistencies in the immune response in normal and pathological conditions, as well as for the identification of the peculiarities of the immune equilibrium maintenance in different conditions.

ORGANIZATION OF THE HUMAN T-CELL REPERTOIRE

Recently, next-generation sequencing technologies and mathematic analysis have expanded the understanding of the clonal organization of the TCR repertoire in humans. It has been shown that the distribution of T-cell clones in the general TCR repertoire complies with the general consistency pattern within the human population and is not age-dependent (22, 40). The distribution of clones in the descending rank order (r) depending on the size of a clone (C) is subject to power-law distribution, *i.e.* the rank (r) of the largest clones correlates with their size (C) according to the power-law distribution $r \sim C^{-\alpha}$, wherein α is a scaling exponent (22, 41). The size of a clone is inversely proportional to its rank, *i.e.* the larger the clone, the lower is its rank, and *vice versa*. This dependence is described by a power-law function $y = kC^{-\alpha}$ (40, 41). Empiric calculation of α is associated with a number of difficulties and depends on the used methods of sequencing and mathematical analysis. Thus, a recent study on the frequency distribution of T-cell clones of two independent cohorts showed a power-law relationship between the rank and size of the largest clones. At the same time, in both cohorts, α was almost similar and was equal to ~1.2 (22). The character of distribution of T-cell clones was similar in people of different ages. Thus, the general TCR repertoire in different people is characterized by a similar frequency distribution of T-cell clones regardless of age and is represented by a small number of dominant clones and a large variety of minor clones, which is consistent with the general type of Pareto distribution (**Figure 1**) (42).

A significant part of dominant clones in healthy people comprises zero insertion clones that are formed before birth and preserve in high abundance for several years with a tendency to a slight decrease throughout life (22, 24). At the same time, these clones provide the basis for public TCR repertoire in different individuals, which raises the issue of the presence of the inborn evolutionary determined set of T-cell clones within the adaptive immunity (11, 22, 24). If these clones exert certain functions or if they are a by-product of the formation of the TCR repertoire in the fetal period, it should be the subject of further studies.

Meier et al. (40) studied the frequency distribution of the TCRβ sequences at each level of the combination of gene segments (DJ, VDJ, and VDJ + NI) and revealed a fractal organization of the TCR repertoire and self-similarity of the frequency distribution of unique TCR clones (**Figure 2**) (40). An earlier study revealed the fractal organization of $CD8^+$ TCR

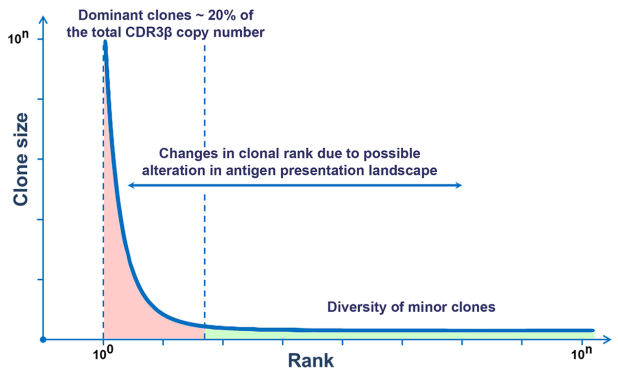

FIGURE 1 | Rank–size frequency distribution follows a power-law distribution. This graph demonstrates the ranking of TCR clones by sizes. To the left, there are few dominant clones (red), and to the right, there is the long tail reflecting a multitude of minor clones (green).

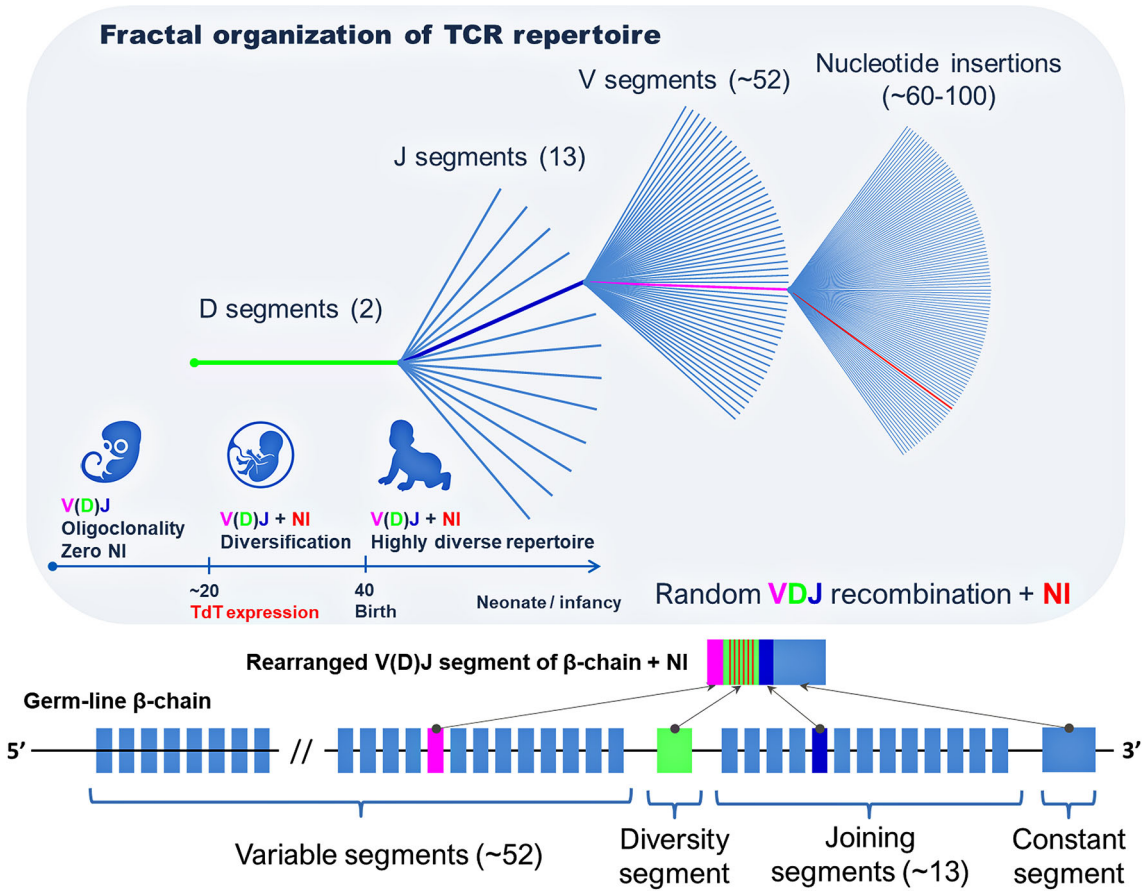

FIGURE 2 | Fractal organization of T-cell repertoire. A fractal is a set with self-similarity (an object that exactly or approximately coincides with a part of itself, similar to itself on any magnification).

repertoires (43). At the same time, persons that are similar by human leukocyte antigens (HLAs) have a similar organization of TCR repertoires, which confirms the involvement of MHC genes in the formation of self-similarity pattern with a strict hierarchy of dominant and minor clones in the individual TCR repertoire (40).

Besides, this study showed that the development of the "graft-*versus*-host disease" (GVHD) in recipients after transplantation of hematopoietic stem cells (HSCs) is associated with the changes in the clonal organization of the TCR repertoire and change of dominant clones within the first four ranks in comparison with the respective donors (40). Probably, such a shift in dominant clones is associated with incomplete identity by MHC genes between donors and recipients. GVHD is based on the incompliance between the landscapes of self-antigen presentation, which leads to the activation and expansion of minor self-reactive clones in recipients with GVHD (40, 44). This can be associated not only with quantitative changes in the clonal organization of the TCR repertoire but also with the plasticity of some subpopulations of T cells and the respective functional changes within these subpopulations (45, 46). In particular, the transition of some Tregs to some subpopulations of the effector cells or polarization of Th0 into Th17 cells in HSC recipients can lead to the development of GVHD (46, 47).

Transdifferentiation between the different T-cell subpopulations is well-known. However, not long ago, it was established that functionally different subpopulations of $CD4^+$ cells expressed TCRs with different physicochemical properties and had different profiles of VDJ recombination, which affected their tendency to differentiate into each other (48). In their study, Kasatskaya et al. (48) focused on some characteristics of the CDR3 region in different subpopulations of T cells. The authors of that study evaluated different properties of amino acids in the CDR3 loop, the hydrophobicity of the loop (Kidera factor 4) (49), the length of the CDR3 loop, the predicted averaged binding energy of the TCR-pMHC (50, 51), and some other parameters that generally influence the affinity of Ag-specific TCR-pMHC interaction and the degree of TCR cross-reactivity (48). The study of these parameters revealed the differences in the physicochemical properties of the CDR3 TCR loop at the level of different subpopulations of T cells. It was shown that Treg cells have TCRs with high cross-reactivity, while follicular helpers Tfh have TCRs with minimal cross-reactivity (48). TCRs of Treg cells exert relatively higher affinity to self-antigens, bind cognate pMHC ligands less specifically and have lower averaged

energy of TCR-pMHC binding than Tfh cells that bind cognate pMHC ligands with high affinity and have a higher energy of TCR-pMHC binding, which agrees with previous data (52–54). At the same time, such differences were also observed in other subpopulations: amino acidic characteristics of the CDR3 loop among the populations Th1/Th1-17/Th17 were similar to the characteristics of Tfh, while among populations Th22/Th2a/Th2, there was a similarity with Treg cells (48). Besides, different subpopulations of T cells were distinguished by a diversity of TCR repertoires. The highest diversity was observed in the subpopulation Tfh. A relatively high TCR diversity was observed in the subpopulations Th2, Th17, Th1, and Treg, while subpopulations Th22 and Th2a had signs of oligoclonal expansion, which indicated Ag-specific proliferation in these subpopulations (48). The physicochemical properties of TCRs are different in naïve TCR T cells and memory T cells, which was shown for populations of $CD4^+$ and $CD8^+$ lymphocytes. At the same time, for naïve nTreg and memory mTreg cells, such differences were not revealed (48, 55). Thus, the functional specialization of T cells depends on the structure of the CDR3 region and could be determined by the interaction of TCRs with the respective diversity of pMHC epitopes for each T-cell population (**Figure 3**). A paired analysis of the overlap of CDR3β diversity of different subpopulations of T cells revealed certain consistency in the transdifferentiation in healthy donors. A high plasticity was revealed between the functionally close populations Th17/Th22, Th17/Th2, Th22/Th2, Th2/Th2a. Lower plasticity was revealed between other populations Th17/Treg and Th1/Th17. At the same time, Tfh and Treg subpopulations were characterized by a high discreteness and had few CDR3β sequences common with other subpopulations of T cells (**Figure 3**) (48). Such data were first obtained for healthy donors and agreed with the data obtained *in vitro* or on animal models (38, 56, 57). Taking into account that the functional specialization of the subpopulation of T cells could be defined by specific interaction between TCR-pMHC and physicochemical properties of the CDR3 loop, the changes in the presentation landscape of antigens can lead to changes in the clonal structure of certain subpopulations of T cells. In some cases, such

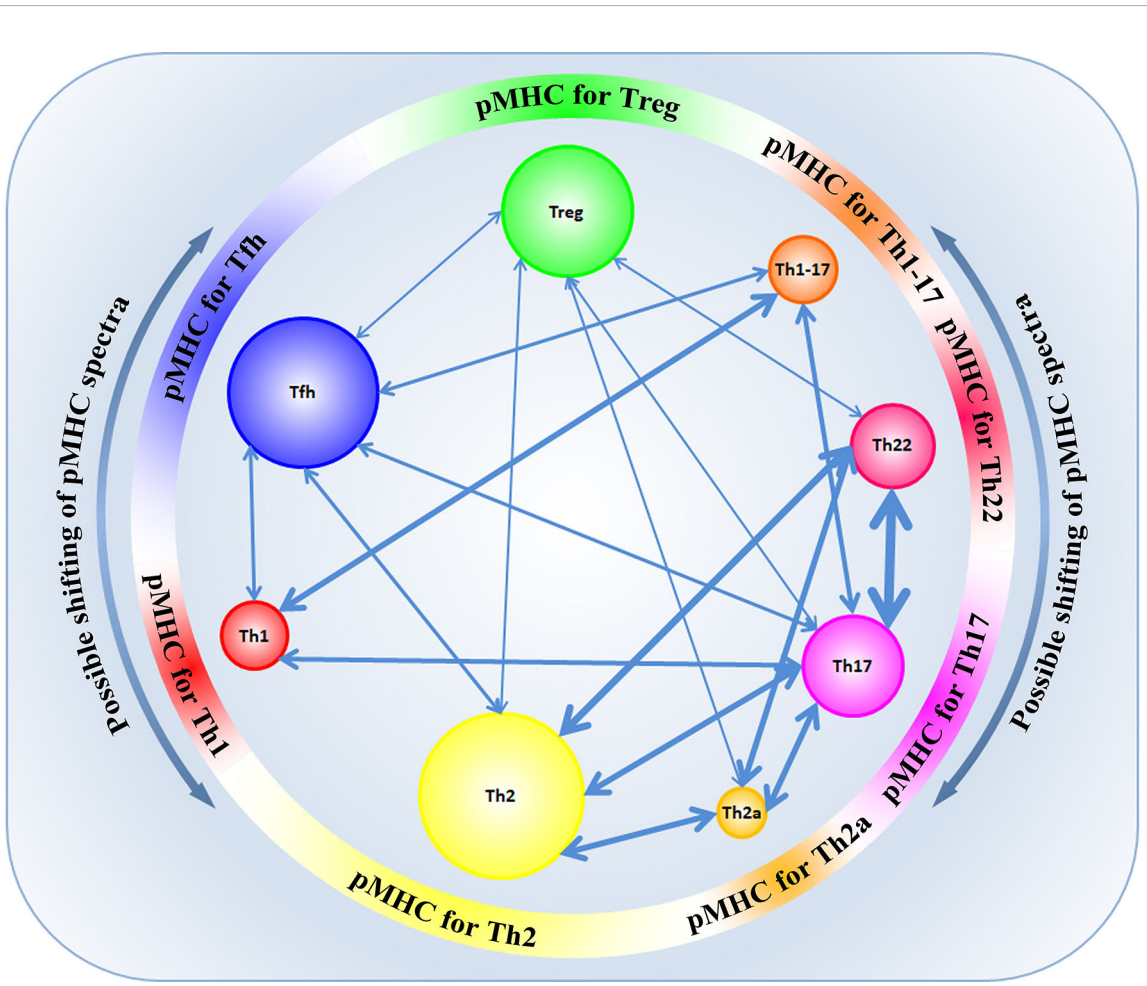

FIGURE 3 | The landscape of presentation shapes different $CD4^+$ subpopulations according to their CDR3 physicochemical properties and specificity. Possible shifts in pMHC spectra can contribute to transdifferentiation between some subpopulations of $CD4^+$ T cells. The width of the arrows reflects the number of common TCR clonotypes between subpopulations.

transitions of Ag-specific clones between subpopulations of T cells are involved in pathological processes. For example, an important role of a transition Treg ↔ Th17 was established in patients with different autoimmune conditions, graft rejection, and oncologic processes (58–63). Besides, plasticity Th17 → Th1 was revealed in patients with juvenile idiopathic arthritis and Crohn's disease. In patients with allergic conditions and bronchial asthma, an enhanced transdifferentiation Th17 → Th2 was observed (64, 65). An imbalance between the subpopulations Th1/Th2 explains the pathogenesis of allergic and some oncologic diseases. However, in this case, an incorrect functional specialization of certain Ag-specific clones in the ontogenesis resulting from the changes in the conditions of presentation of the respective antigens is observed rather than a transdifferentiation (66–68).

Similar to $CD4^+$ lymphocytes, an imbalance between effector $CD8^+$ Teff and regulatory $CD8^+$ Treg cells is significant for the maintenance of the immune equilibrium. Along with humoral factors of suppression, $CD8^+$ Treg cells can exert Ag-specific suppressive activity mediated by the interaction with antigen-representing cells (69). Shifts in the represented antigen spectra and changes of conditions of their presentation can contribute to the irrelevant $CD8^+$ Teff ↔ $CD8^+$ Treg transdifferentiation (70). It was shown that such plasticity between subpopulations of $CD8^+$ lymphocytes significantly contributed to the pathogenesis of different autoimmune and infectious diseases and oncological processes and took part in the graft rejections (69, 71–73). Still, despite the present achievements in the understanding of the organization of the T-cell repertoire, the identification of certain clones involved in the pathogenesis of different diseases attracts the attention of scientists in modern immunology and opens perspectives for personalized medicine.

ORGANIZATION OF THE HUMAN B-CELL REPERTOIRE

The immunoglobulin gene rearrangement of B cell in the bone marrow results in the formation of a highly diverse repertoire of naïve (antigen-inexperienced) B cells that get into the peripheral circulation (74, 75). Similar to T cells, this process occurs under the influence of a complex of RAG, TdT, and a number of enzymes. Their activation induces V(D)J recombination and P- and N-insertions in the CDR3 loop of naive B cells (75–78). Further diversification of the BCR repertoire is associated with SHMs that occur in a cell under the influence of activation-induced deaminase (AID) in the peripheral lymph nodes with a cognate antigen (79, 80). This process underlies the affinity maturation of antibodies and targets an increase in the specificity of antigen recognition (75). Similar to T cells, the frequency distribution of clones in the general repertoire of B cells complies with power-law distribution (**Figure 1**) and agrees with the general biological type of Pareto distribution (81, 82). In other words, the BCR repertoire contains a relatively low number of dominant clones and an extremely high diversity of minor clones that form a long tail of distribution. Besides, as for T cells, the repertoires of naïve B cells are characterized by a tree-like fractal organization (**Figure 4**) (82, 83).

However, the architecture of the BCR repertoire has some peculiarities that are closely associated with the process of SHM and the formation of memory plasma cells. The diversity of the repertoire of these cells is significantly lower than the diversity of naïve B cells (84), which is associated with the history of antigen challenges that an organism faces throughout its life. At the same time, a tree-like structure of the repertoire of naïve B cells, which is generated due to VDJ recombination and nucleotide insertions, is replaced by a star-like structure for memory B cells and plasma cells, which is associated with the process of SHM (**Figure 4**) (82, 85). Such star-like structures reflect the process of activation of one or several B-cell clones closed by specificity. In the course of further expansion and SHMs, these B-cell clones form a set of antigen-experienced B-cell clone neighboring in the common space of CDR3 sequences. Part of these cells later becomes plasma cells. In this case, for the evaluation of similarity/closeness of the clones, Levenshtein distances were used (82, 86). Finally, the activity of AID is capable of mediating the shift of heavy chains from IgM/IgD to IgG, IgA, or IgE during SHM (74). It should be noted that despite the high personalization of BCR repertoires, different individuals have a similar organization of BCR and antigen repertoires (82, 87), which indicates general principles of the formation of BCRs and Ig diversity in different individuals. At the same time, the diversity of naïve B cells is affected by self-antigens and the repertoire of naïve B cells is limited by positive and negative selection (35, 36). The diversity of plasma cells directly depends on the diversity of antigen challenges within the life and depends on the functional activity of T cells, which is confirmed by a significant decrease in SHM in T cell-deficient mice (36, 87).

It is well-known that B cells play a central role in humoral immunity as antibody producers, can express some cytokines, and act as antigen-presenting cells (88–90). During the past years, many studies have been dedicated to the subpopulation of B cells with regulatory functions that were called B regulatory cells (Bregs) (91–94). Bregs exert their functions due to the production of anti-inflammatory cytokines, inhibit different populations of immune cells, and can induce the formation of Tregs from effector T cells acting as tolerogenic antigen-presenting cells, which do not exclude the Ag-specific effect of Bregs (91, 95, 96). Similar to T cells, irrelevant induction of Bregs and an imbalance between effector and regulatory B cells play a significant role in the pathogenesis of different autoimmune and oncologic processes, in patients with chronic infections and graft rejections (97–101). However, in this case, a transition Beff ↔ Breg could be primarily associated with the peculiarities of the microenvironment and only indirectly mediated by the shifts of spectra of the presented antigens and the conditions of their presentations *via* T cells (91, 102–104). Still, in some cases, the induction and functional activity of Bregs depend on the recognition of cognate antigens by Breg cells; and the suppression activity of Bregs can be mediated by direct B–T cellular interaction, which confirms the possibility of the Ag-specific effect of Bregs (105, 106). Thus, the conditions of the microenvironment and spectra of B-dependent antigens in

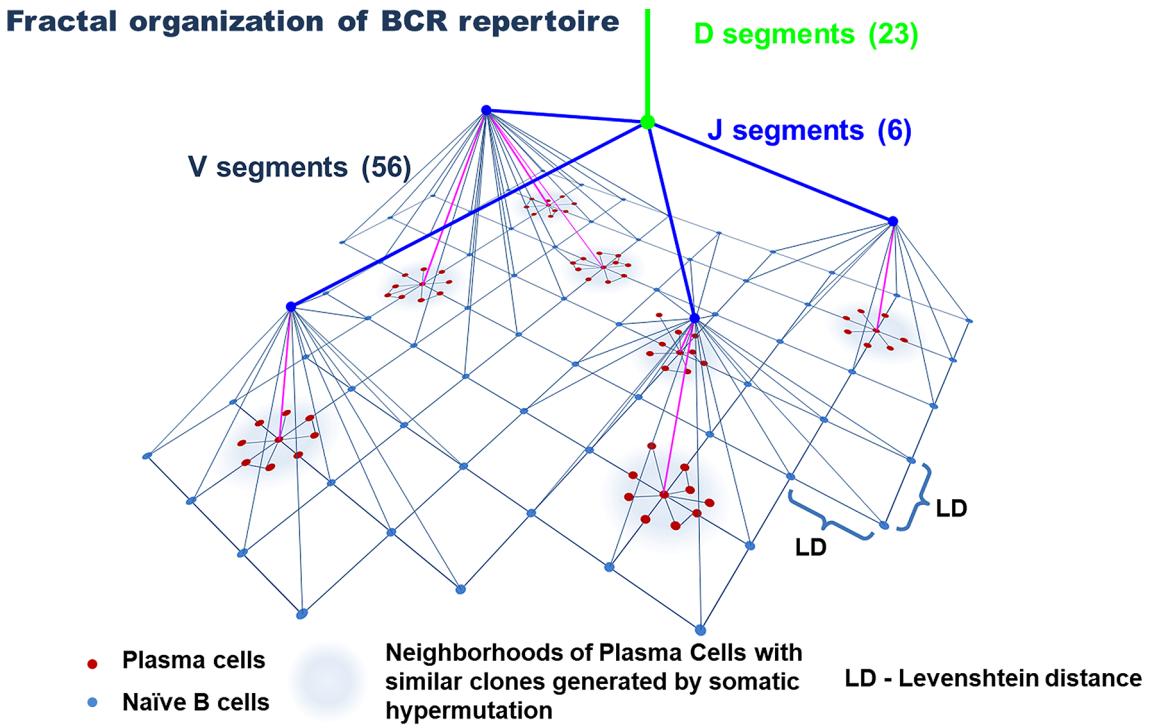

FIGURE 4 | Model of the organization of the B-cell repertoire. Tree-like structures generated by VDJ recombination and nucleotide additions/deletions and a star-like structure for plasma cells likely generated by somatic hypermutation. The uniform distribution of naive B cells in the similarity layer schematically reflects a homogeneously interconnected network (by Levenshtein distances) of these cells, in contrast to plasma cells, that form highly interconnected subnetworks of similar clones. The number of V, D, and J segments is indicated for the IgH chain.

the microenvironment of B cells influence their functional specialization; and an irrelevant transdifferentiation Beff ↔ Breg can underlie the pathogenesis of different pathologies.

An extremely high diversity of Ag-recognizing receptors of T and B cells provides the formation of qualitatively new properties that distinguish adaptive immunity from innate. The most important of them is the specificity of antigen recognition. Another important property is universality, *i.e.* the adaptive immune system can potentially specifically recognize any antigen of all possibilities. Redundancy—the same antigen can be recognized by different Ag-recognizing receptors due to cross-reactivity and the fact that one antigen can have different epitopes. Clones that are close by their specificity can duplicate and replace each other during the formation of the immune response. This underlies the robustness of adaptive immunity. In general, these properties provide the reliability of the immune system functioning and reflect the qualitative–quantitative transition, when a high diversity of Ag-recognizing receptors provides a qualitatively new level of the immune system functioning. However, changes in the conditions of antigen presentation can lead to situations when a disturbance of functional specialization of some subpopulations of T and B cells occurs, which can underlie the pathogenesis of different pathologies and be the main factor in the disturbances of the adaptive immunity.

PRESENTATION LANDSCAPE

Rearrangement of a genome underlies a colossal diversity of Ag-recognizing receptors (17, 75, 107). However, the final formation of naïve repertoires of T and B cells is observed during the process of positive, and then, negative selection and depends on the diversity of the antigens presented in the thymus (108, 109) and the diversity of self-antigens in bone marrow (110, 111). In the past years, more data have been accumulated that confirm the influence of allele variants of MHC on the formation of individual TCR landscape (25–27, 112, 113).

T-CELL PRESENTATION LANDSCAPE

The significance of MHC restriction for the development of T cells is well-known and can be illustrated by a recent study. It showed that during positive and negative selection, a selection of T cells with certain properties of the CDR3 loop occurred (114). Thus, during the process of positive selection, MHC restriction provides the selection of TCRs with the length of CDR3 (8–13 amino acid residues) and limits the selection of TCRs with positively charged and hydrophobic amine acid residues in the CDR3 loop. During the process of negative selection, it prevents the selection of TCRs with the residues of cysteine in the Ag-binding regions of the CDR3

loop (114). Probably, in this case, the selection of TCRs is primarily influenced by the physicochemical properties of MHC molecules and not certain epitopes in their composition. It should be noted that MHC restriction does not lead to the selection of TCRs with certain sequences of amino acids in the CDR3 loop and preserves high randomness of amino acid sequences in the CDR3 loop and sufficient diversity of TCRs for the recognition of the variety of potential antigens (9, 114, 115).

Apart from the common physicochemical properties of MHC molecules, an important role in the formation of the naïve repertoire of TCRs is played by epitopes presented in the thymus as a part of MHC molecules. The formation of the central auto-tolerance occurs due to the independent activity of transcriptional factors AIRE and Fezf2 that induce the expression of different tissue-restricted antigens by medullary thymic epithelial cells (mTECs) and thymic B cells (AIRE), which provide the elimination of self-reactive T cells during negative selection (109, 116–119). At the same time, some T cells, that exert a relatively high affinity to self-antigens, become Treg cells (38, 117). The affinity of TCR Treg cells to self-antigens is 100-fold lower than in the self-reactive T cells that undergo negative selection (38, 120). It was established that the transcriptional factors AIRE and Fezf2 provided the expression of nearly 60% of tissue-restricted antigens presented in the thymus (116). It is suggested that other antigens are presented in the thymus by different subpopulations of dendritic cells (DCs) (121). Presently, at least three DC subpopulations are known to provide the presentation of antigens in the thymus: CD8α^+, Sirpα^+, and B220$^+$ plasmacytoid dendritic cell (pDCs). CD8α^+ DCs occupy ~50% of the pool of thymic DCs (121), develop from the precursors in the thymus (122), and present tissue-restricted antigens, obtained from mTECs during trogocytosis or uptake of extracellular matrix, to T cells (123). Sirpα^+ DCs occupy ~20% of the pool of thymic DCs, are found primarily in the corticomedullary perivascular spaces (124), and present antigens taken up from the bloodstream or acquired in the peripheral tissues before the migration to the thymus (121). The remaining ~30% of the pool of thymic DCs represent B220$^+$ pDCs and present antigens obtained primarily on the periphery before the migration to the thymus (121). The presentation of self-antigens by the thymic DCs provides a negative selection of self-reactive CD4$^+$ and CD8$^+$ cells and probably contributes to the formation of Treg cells. However, precise mechanisms of this process are understudied (121, 125, 126). It is suggested that the migration of DCs from the periphery and the pathologic migration of B cells to the thymus (127) create the risks of inadequate formation of the central tolerance, for example, to the tumor or infectious antigens. However, this assumption requires experimental confirmation. Besides, it should be noted that the mechanisms of central tolerance do not exert absolute efficiency, and some self-reactive T cells can get to the peripheral bloodstream, which should be suppressed by Treg cells in normal conditions (54). This creates additional risks for the development of autoimmune diseases.

Thus, as a result of the described process, two varieties of T cells are formed that leave the thymus: T cells with relatively high affinity to self-antigens (Tregs) and T cells with low affinity to self-antigens (future anti-pathogen T cells) (54). After leaving the thymus, recent thymic emigrants (RTEs) migrate to the peripheral lymph nodes. During the maturation under the influence of the microenvironmental factors, they form a variety of mature naïve T cells (128). It is suggested that on the periphery, RTEs are subject to additional selection that is provided by MHC molecules in the peripheral lymph nodes. Only some RTEs with certain physicochemical properties of the CDR3 loop become mature naïve T cells (128). Probably, this stage of peripheral selection is necessary for the culling of potentially self-reactive T cells that manage to avoid negative selection in the thymus and deletion of T cells that cannot recognize effectively MHC molecules (48, 55, 128). At the same time, it is suggested that Treg cells do not undergo this stage of additional peripheral selection, which is confirmed by the lack of differences in physicochemical properties of the CDR3 loop between immature and mature subpopulations of Treg cells (48). Thus, the presentation landscape on the periphery provides an additional stage of the selection of CD4$^+$ and CD8$^+$ cells. As a result, a formation of the repertoire of mature naïve T cells occurs that will further go through a functional specialization according to their physicochemical properties and the specificity of their TCRs and form the main subpopulations of Th lymphocytes (48, 55, 129–131). It is evident that the changes in the landscape of presentation of antigens associated with different antigen challenges in the ontogenesis will influence the further formation of the TCR repertoire and clonal organization of different subpopulations of T cells in a certain individual. At the same time, it should be noted that the personalization of TCR repertoires of CD4$^+$ and CD8$^+$ is significantly provided by the individual set of allele variants of MHC genes (26, 31–33, 112).

GENETIC VARIANTS OF MHC MOLECULES INFLUENCE THE LANDSCAPE OF RECOGNITION

During the past decade, a lot of attention has been paid to the study of the influence of MHC allele variants on the repertoire of TCRs in different individuals. Associations were revealed between MHC genetic variability and the profiles of expression of TCR V genes (132). It was established that such associations were provided not only by the contact of TCRs with a peptide in the MHC complex but also by a physical contact between V-regions of the TCR β-chain and complementary regions of MHC molecules (132, 133), which indicated a direct influence of MHC genotypes on the formation of individual TCR repertoires. This agrees with the assumption of Niels Jerne on the co-evolution of MHC and TCR genes for a better predisposition to interact with each other (134). Still, the influence of the MHC genotype is primarily observed on the CD8$^+$ lymphocytes. This is explained by a closer contact between TCRs of CD8$^+$ cells and MHC-I molecules, while the regions CDR1 and CDR2 in TCRs of CD4$^+$ cells have a weaker contact with complementary regions of the MHC-II molecules, and the region CDR3 primarily contacts with a peptide in the MHC-II complex (133, 135). This could provide

the lack of influence of individual polymorphism of MHC-II genes on the diversity of the $CD4^+$ repertoire, while a higher polymorphism of the MHC-I gene in heterozygotes is associated with a higher diversity of $CD8^+$ cells (27). Still, a recent study on animal models showed a direct influence of MHC-II allele variants on the diversity and clonal organization of the $CD4^+$ repertoire, including Treg cells (112). This confirms the hypothesis that allele variants of MHC-I and MHC-II play an important role in the formation of TCR repertoires of $CD4^+$ and $CD8^+$, respectively. Besides, it was shown that mutations in the conservative regions of MHC-I and MHC-II that contact with the complementary regions of TCRs influence the profiles of expression of TRAV and TRBV in the $CD4^+$ and $CD8^+$ repertoires and change their clonal organization (136, 137).

Thus, it is evident that the individual HLA phenotype defines epitope spectra that could be presented with the highest possibility, *i.e.* HLA phenotype is responsible for the formation of immunopeptidomes of MHC-I and MHC-II-associated antigens (138, 139). In turn, this affects the selection of T cells in the thymus, plays an important role in the formation of individual TCR repertoires on the periphery, and determines the individualization of the immune response. Such association between HLA genes and TCR repertoires reflects a close functional and phylogenetic association between innate and adaptive immunity.

POTENTIAL RISKS OF IMMUNE DISEQUILIBRIUM

The equilibrium in the immune system is achieved due to fine coordination between the innate and adaptive branches of immunity. Potential risks of the immune disequilibrium can be associated with different genetic factors, all possible antigenic challenges, and the influence of unfavorable factors of the environment.

It is well-known that there is a genetic predisposition to autoimmune diseases (ADs). It is hypothesized that the influence of different HLA variants and other genes associated with ADs in combination with epigenetic factors and unfavorable exogenous conditions contributes to the development of ADs (140). However, the presence of genetic predisposition does not always lead to the realization of the risk of ADs. Probably, an additional trigger is required (long-term lymphopenia or some immune regulatory disturbance) (141, 142). During the past two decades, the role of homeostatic proliferation in the development of ADs has been widely discussed (54, 143–147). This is a physiological process of the quantitative restoration of the peripheral pool of T cells after lymphopenia of any etiology by means of the antigen-specific proliferation of lymphocytes under the influence of IL-7 and IL-15 that could acquire pathological traits depending on the depth of lymphopenia (54, 148, 149). It was shown that this process could result in the selection of potentially self-reactive clones of T cells due to the competition for the contact of TCRs with self-pMHC, in a decrease in the diversity of the general TCR repertoire, and in a decrease of functional activity of Treg cells because of the deficiency of IL-2 in the conditions of lymphopenia (54, 150–153). Besides, a disturbance of the functional specialization of Treg cells and their conversion into pathogenic Th lymphocytes can occur (154). Some studies showed that Treg cells could not suppress the proliferation of T cells that received a strong TCR signal under the influence of IL-7 and IL-15, which was important in the context of homeostatic proliferation when a strong TCR signal gives advantages to T cells in the competition for the factors of survival (155, 156). This fact is interesting taking into account that AD-associated variants of MHCs contribute to a better presentation of antigens associated with the disease, and thus, homeostatic proliferation can contribute to the expansion of self-reactive T-clones in people with genetic predisposition (157, 158). Thus, a shift of spectra of the presented antigens towards self-antigens that are provided by AD-associated HLA variants and mediated *via* homeostatic proliferation of the changes in the clonal organization of TCR repertoires can underlie the disturbances in the immune equilibrium in patients with ADs.

Homeostatic proliferation can lead to a favorable antitumor immune response (159, 160). This response is formed as a result of polyclonal homeostatic expansion in the lymph nodes and is characterized by $CD8^+$-cell cytotoxicity, an increase in the concentration of IFNγ, and the formation of memory cells (159). Besides, some data indicate that the shift in focus of homeostatic proliferation from $CD8^+$ to $CD4^+$ cells can be one of the causes of the development of ADs (159, 161). At the same time, the homeostatic proliferation of B cells does not lead to negative effects because it is exerted *via* an Ag-independent pathway and does not influence the diversity and clonal organization of the BCR repertoire (162, 163).

Probably, homeostatic proliferation can also contribute to a decrease in the general diversity of TCRs and the TCR diversity of naïve T cells with age (23), which negatively affects the protective function of the immune system against infections or other antigenic challenges in senior age (23, 164). It was shown that increased sensitivity to viral and oncological disease was associated with a decrease in the diversity of TCRs and connected with the formation of holes in the TCR repertoires (164–167).

The conditions of the microenvironment can significantly affect the functional activity of T and B cells causing their activation or leading to anergy and inducing the formation of Treg and Breg cells. An inflammatory microenvironment and co-stimulating signals that are transmitted during contact with the neighboring cells can lead to non-specific activation of different lymphocyte clones due to a so-called bystander effect (168). Since self-antigens can be present in the site of inflammation or immune response to an infection and any other antigen, the bystander effect can potentially cause unfavorable activation of self-reactive clones and increase the risk of ADs (168–171). Probably, a functional modulation of the TCR activation threshold due to the factors of co-stimulation and inflammatory microenvironment can contribute to the non-specific activation of T cells (172, 173). It was established that as a result of the bystander effect, a disturbance in the functional specialization of different subpopulations of lymphocytes could occur, for example, a transition of Treg cells into pathogenic Th

lymphocytes, which could be also associated with the risk of the development of ADs (174). In other words, the bystander effect can lead to irrelevant Ag-independent activation of self-reactive lymphocytes and their expansion changing the structural organization of TCR and BCR repertoires at the level of separate clones and can contribute to the development of ADs.

Apart from the activation of lymphocytes, the factors of the microenvironment can cause anergy or even induce lymphocytes with regulatory functions by the Ag-independent bystander suppression. Similar effects are observed in the microenvironment of tumors that express some suppressor factors forming a tolerogenic medium and avoiding the immune surveillance (175, 176). At the same time, in the microenvironment of the tumor, a population of tolerogenic dendrite cells is formed. These cells are responsible for the formation of tumor-specific tolerance that is provided by T and B lymphocytes with regulatory functions (177–180). Besides, there is a possibility of the formation of central tolerance due to a migration of some dendrite cells loaded with tumor antigens to the thymus, wherein they can potentially be involved in the process of negative selection of T cells (121, 124–126). However, this assumption is hypothetical, and this issue requires additional research. Thus, the changes in the conditions of presentation of tumor antigens can shift the immune response from the immunogenic to tolerogenic and result in the respective changes in the clonal organization of T- and B-cell repertoires (181, 182). Besides, it changes the functional specialization of different lymphocyte populations providing the progression of the tumor growth.

CONCLUSION

The maintenance of the equilibrium in the immune system is an intricate dynamic process associated with constant changes in the landscapes of presentation and recognition, wherein genetic HLA variants play an important role, influence the formation of TCR repertoires, and determine the individualization of the immune response. In general, the disturbance of the immune equilibrium (autoimmune, infectious, or oncogenic process) is associated with the changes in the conditions of presentation and the spectra of the presented antigens, as well as with the transformation of T- and B-cell repertoires and a shift in the functional specialization of some T and B cells. In this case, the most important role is played by the genetic background and the influence of external environmental factors.

It is suggested that the study of genetic HLA variants and immunopeptidomes associated with a disease in a certain individual and the identification of certain clones of T and B cells involved in the pathogenesis of the disease will allow using personalized approaches to the therapy of different pathologies based on a targeted, specific effect on certain pathology mechanisms.

AUTHOR CONTRIBUTIONS

DS contributed to the conception, drafting of the manuscript, and design. VT contributed to the conception and revision. VK contributed to the revision and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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The Role of Regulatory T Cells in Pulmonary Arterial Hypertension

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Pulmonary arterial hypertension (PAH) is a chronic, incurable condition characterized by pulmonary vascular remodeling, perivascular inflammation, and right heart failure. Regulatory T cells (Tregs) stave off autoimmunity, and there is increasing evidence for their compromised activity in the inflammatory milieu of PAH. Abnormal Treg function is strongly correlated with a predisposition to PAH in animals and patients. Athymic Treg-depleted rats treated with SU5416, an agent causing pulmonary vascular injury, develop PAH, which is prevented by infusing missing $CD4^+CD25^{high}FOXP3^+$ Tregs. Abnormal Treg activity may also explain why PAH disproportionately affects women more than men. This mini review focuses on the role of Tregs in PAH with a special view to sexual dimorphism and the future promise of Treg therapy.

Keywords: regulatory T cell, pulmonary arterial hypertension, sexual dimorphism, right ventricle, estrogen

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INTRODUCTION

The first classification of pulmonary hypertension (PH) was proposed in 1973 and is now defined as an increase in mean pulmonary arterial pressure (mPAP) > 20 mmHg at rest, normal left atrial pressure and pulmonary vascular resistance >= 3 Wood units (1). This review focuses on PAH which is classified by World Health Organization (WHO) as Group 1 PH (1). PAH includes idiopathic or sporadic cases (IPAH), heritable cases (HPAH, describing patients with a family history or identified germline mutations), and associated forms (attributable to conditions like anorexigens, liver diseases, congenital heart diseases, and connective tissue diseases) (2). Heterozygous germline mutations in gene encoding for bone morphogenetic protein receptor 2 (BMPR2) account for 53-86% of the familial cases of PAH and 14-35% of patients with IPAH (3). Although clinical features of HPAH and IPAH are indistinguishable, patients with HPAH have an earlier disease onset with more severe hemodynamics (4). There is a strong sex-associated influence on the development of PAH, with an approximate female to male patients' ratio of 4:1 (5). Although the incidence of PAH is higher in women, the estimated 5-year survival is less favorable in men (52% in men compared to 62% in women) (6). Regardless of the underlying etiology, patients with PAH exhibit similar pathological alterations including remodeling of the pulmonary arterioles driven by proliferation/dysfunction of both pulmonary arterial endothelial cells (PAECs) and smooth muscle cells (PASMCs), *in situ* thrombosis, increased pulmonary arteriole contractility, and enhanced inflammatory infiltrates (7–9).

PAH is a lethal cardiopulmonary condition characterized by pulmonary vascular remodeling and right heart failure (10). Drugs used to treat this disease primarily target pathologic vasoconstriction, but there is an additional rationale for treating immune dysregulation (9). Growing evidence indicates that abnormal Treg activity promotes the development of PAH, and accentuating the activity of these cells has therapeutic potential (9). Tregs play a vital role in maintaining immune homeostasis, fostering tissue regeneration, and limiting vascular injury (11–13). They protect against autoimmunity by dampening inappropriate responses to self-antigen (14). This defensive activity was first identified in mice lacking thymic-derived $CD4^+CD25^{hi}$Forkhead box P3 $(FOXP3)^+$ Treg cells and in Treg-depleted animals which develop a constellation of autoimmune conditions including thyroiditis, diabetes, autoimmune gastritis, and inflammatory bowel disease (14, 15). Subsequently, numerous pre-clinical autoimmunity models show that Treg defects are implicated in the development of autoimmune diseases and prevented by the adoptive transfer of Tregs (14). Other types of $CD4^+$ regulatory T cells are also discovered including IL-10-producing type 1 regulatory T cells and TGF-β producing-T helper 3 cells (16, 17). Here, we only discuss the $CD25^{high}FOXP3^+$ Treg population given the broader and indispensable roles of these cells.

PAH-associated conditions exhibit Treg abnormalities (18). Furthermore, Treg dysfunction in PAH affects males and females differently and may arise because of pathogenic gene variants (e.g., mutations in *BMPR2* and chronic vascular inflammation (18–20). Our group demonstrated that adoptive transfer of Tregs is effective in preventing pre-clinical PAH (21). Treg infusions restore immune regulation, reduce endothelial injury, impede PAH-mediated vascular remodeling, and prevent right heart failure (21, 22). Because Tregs can limit and potentially reverse pulmonary vascular disease, Treg infusions may become a viable treatment for this condition (23). This review explores how genetic and environmental cues may cause Treg abnormalities that exacerbate PAH and discusses how the restoration of Treg function may attenuate this life-threatening disease.

REGULATION OF TREG ONTOGENY, IDENTITY, AND FUNCTION

Anti-inflammatory $CD4^+CD25^{high}FOXP3^+$ Tregs comprise approximately 5% of $CD4^+$ T cells and develop primarily in the thymus (24–27). Genetic mutations (e.g., *FOXP3*, *RAG1*, and *AIRE*) (28) impact Treg thymic development and predispose individuals to autoimmune conditions. Continued expression of FOXP3 and sustained FOXP3 signaling are required for Tregs to maintain their lineage-stability and function, and FOXP3 expression is influenced by local tissue microenvironment and sex hormones (26). For example, FOXP3 may be turned on in conventional T cells (Tconvs, $CD4^+FOXP3^-$) at peripheral inflammatory sites to promote Treg phenotype (pTregs) (29). High concentrations of interleukin-1 beta (IL-1β) as well as interleukin-6 (IL-6) may result in decreased FOXP3 and increased interleukin-17 (IL-17) expression in Tregs (exTregs) (30–32). Specifically, proinflammatory IL-6, in conjunction with IL-1 and IL-23, induces the expression of RORγt and IL-17 and suppresses FOXP3 thereby causing a genetic reprograming in $FOXP3^+$ Treg cells (33). Elevated IL-6 also promotes the methylation of a conserved Cp-G-rich island of *FOXP3* gene and results in reduced gene transcription (34). Notably, IL-1β, IL-6, and IL-17 are elevated in PAH and contribute to Treg instability and pathogenic inflammation (35, 36). Additionally, Tregs can specialize into T helper-(Th-) like subsets expressing Th1, Th17, Th2, or Th22 markers and receptors that mimic and suppress Th1, Th17, Th2, or Th22 immunity, respectively (37).

Abnormalities in thymic development and Treg production may be related to the most common genetic mutations in PAH involving *BMPR2* (38). Normal thymus development requires bone morphogenic protein (BMP) signaling (39); thymic epithelial cell maturation requires BMP4 (a BMPR2 ligand) and the activation of *FOXN1* transcription factor (40). Consequently, *BMPR2* mutations may affect embryonic thymic BMP4 signaling and influence Treg development in early life. It is unknown whether patients with BMPR2 mutations have decreased Tregs or whether the impact of BMPR2 signaling may be more subtle, not affecting Treg numbers globally but rather subset percentages and function. In peripheral lymphoid tissues, BMPR1α sustains the expression of FOXP3 in pTregs and is required for the maturation and preservation of Treg cell phenotype while inhibiting the differentiation of pro-inflammatory Th1 and Th17 cells (41). Missense mutations of BMPR1β were identified in pediatric IPAH cases (42) suggesting that BMPR1β insufficiency may contribute to the imbalance between Treg and Th1/Th17 populations in PAH. Genetic and environmental factors that govern Treg development and functionality (43) may ultimately play a role in the predisposition to PAH after vascular injury.

PAH AND TREG ABNORMALITIES

The immune dysregulation observed in PAH may be attributable to Treg anomalies (9, 18). Tregs maintain immune homeostasis, and their deficiency predisposes individuals to autoimmune injury (13, 26, 44, 45). Just as failing Tregs are increasingly recognized in cardiovascular diseases, abnormalities in Treg number and function are also reported in PAH (18, 36, 46–49). Inflammatory pathologies are associated with poor clinical outcomes in PAH and underscore the importance of understanding Treg derangements (9). Extensive pre-clinical evidence from our group and others suggest that abnormal Treg activity may explain autoimmune and inflammatory features noted in PAH (**Table 1**). Treg numbers are reduced in PAH lungs but increased in the peripheral circulation, displaying reduced suppressive functionality (46, 47, 77, 78). In addition to the thymic anomalies, defective leptin and adiponectin signaling may also influence Treg function in IPAH patients (46, 79).

The heterogeneous vascular lesions of PAH reveal numerous inflammatory cell types in and around the pulmonary vessels,

TABLE 1 | Clinical and pre-clinical studies show a relationship between Treg abnormalities and PAH-associated conditions.

	PAH-associated conditions	PAH prevalence	Treg (T cell) abnormalities
Clinical Studies	Scleroderma	≈10% (50)	↓Treg function and frequency (51)
	Lupus erythematosus	3%-23% (52)	Altered Treg frequency, ↓Treg/Teff(effector T cells) ratio correlate with disease severity (53)
	Sjögren's syndrome	≈10% (54)	Altered Treg frequency; ↓Treg/Th17 (55)
	Polymyositis	≈10% (56)	↓Treg frequency and ↓Treg/Teff ratio (57)
	Antiphospholipid syndrome	Common (58)	↓Treg frequency and ↓Treg/Teff ratio (59)
	Hashimoto's thyroiditis	case reports (60)	↓Treg/Teff ratio and ↓Treg function (61)
	HIV infection	0.5% (62)	Altered Treg frequency, phenotype, and function (63)
	Schistosomiasis	8%-25% (64)	Altered Treg frequency (65)
	Herpesvirus 8	≈3% (66)	Altered Treg frequency and function (67)
	DiGeorge syndrome	case reports (68, 69)	Abnormal thymic Treg development (70)
	IPEX syndrome	case report (ATS) (71)	FOXP3 mutations and Treg defects (72)
	APS-1	case report (73)	AIRE mutations and Treg defects (74)
	Idiopathic PAH	n/a	↓Treg/Th17 (75)
	CTD-PAH	n/a	↓Treg frequency, ↓Treg/Teff ratio and ↓Treg function (36)
Pre-clinical	Athymic rats	100% after SU5416	↓Treg causes PAH (w/o hypoxia), Treg reconstitution protects rats from PAH
	B6 mice	100% after hypoxia	Treg infusion protects mice against PAH (76)

suggesting a coordinated immune response (9, 80). Treg abnormalities may promote the development and progression of PAH by failing to quell this inflammation following vascular injuries, such as that induced by shear stress, hypoxia, ischemia, or pathogens; the result, a prolonged and destructive period of vascular wound healing (9, 18, 20, 21, 81, 82). Beyond the regulation of adaptive immunity, Tregs control innate responses following injury. In a Treg-deficient model of PAH, we show that the absence of Tregs contributes to the emergence of destructive macrophage-based immunity culpable in progressive endothelial damage and vascular remodeling (83). Tregs can also control neutrophilic infiltration and preserve endothelial barrier function, possibly relevant to the pathogenesis of PAH (84, 85).

MODELING TREG BIOLOGY IN PAH

Aberrant Treg activity predisposes animals to vascular inflammation and PAH (86). We initially noted a predisposition to severe PAH in animals lacking normal T cells (athymic rats) after exposure to SU5416, a vascular endothelial growth factor 2 (VEGF2) receptor antagonist that causes pulmonary arteriole injury. [Rats robustly model PAH in a manner not uniformly observed in mice (87)]. Treg-deficient rats exposed to SU5416 demonstrate an accumulation of B cells and macrophages after one week before developing hemodynamically-significant PAH (21). Restoring missing $CD4^+CD25^{hi}FOXP3^+$ cells through intravenous infusion limits inflammation, prevents endothelial apoptosis, and ameliorates PAH in this model; these original studies demonstrate a relationship between Treg deficiency and the proclivity for PAH. In SU5416-treated athymic rats, activated macrophages expand and produce leukotriene B_4 (LTB_4) in a lung environment lacking Tregs. This eicosanoid may contribute to pulmonary vascular disease by inducing endothelial cell apoptosis/transformation, smooth muscle cell proliferation/hypertrophy, and adventitial fibroblast proliferation/migration (83, 88). Interestingly, unlike some animal models which observe PAH more consistently in males, the Treg-deficient rat model shows a profoundly heightened vulnerability to disease in both sexes. In the future, other PAH models (currently in development), with more subtle Treg deficits than the athymic rat, will provide greater opportunities to understand the unique participation of Tregs in pulmonary vascular health and disease.

TREGS AND SEX-RELATED PATHOLOGY IN PAH

PAH disproportionately affects women more than men (89). Our recent athymic rat study highlights the effect of sex on Treg activities in PAH and suggests the Tregs' dominant role in protecting females against this condition (22). Treg sexual dimorphism is a complex subject. While males have a larger thymus, higher Treg numbers, and more robust Treg immune-suppressive capacity, female hormones enhance Tregs' growth and function (90, 91). Because PAH is worse in females in this Treg-deficient model, estrogen (17b-estradiol; E_2) may be exacerbating immunity in the absence of normal Treg function. E_2 is a highly pleiotropic hormone for immune function, being both pro-inflammatory and anti-inflammatory under different conditions and with varying types of cells (92). E_2 promotes Treg differentiation (93–96) while also enhancing Th2 responses (97) and B cell/macrophage activation (98–100). Treg suppressive activity involves E_2-dependent expression of the anti-inflammatory checkpoint molecule Programmed Death-1 (PD-1) (96). Cumulatively, these findings raise the seemingly divergent possibilities that E_2 may promote protective Treg function and that E_2 can intensify harmful immunity (**Figure 1**). Because E_2 supplementation may be a possible therapeutic strategy in PAH (84), it is crucial to discern the molecular underpinnings of E_2 in PAH immune injury. Finally, PAH is prevalent in obese women (101). Tregs are decreased in adipose tissue, a phenomenon that may contribute to persistent low-grade inflammation. Reduced Tregs, expressing adiponectin receptor 1, in the lungs of obese mice promotes inflammation and a predisposition to PAH (79).

In the absence of Tregs, female PAH rat lungs exhibit worse hemodynamics, increased macrophage infiltration, a more

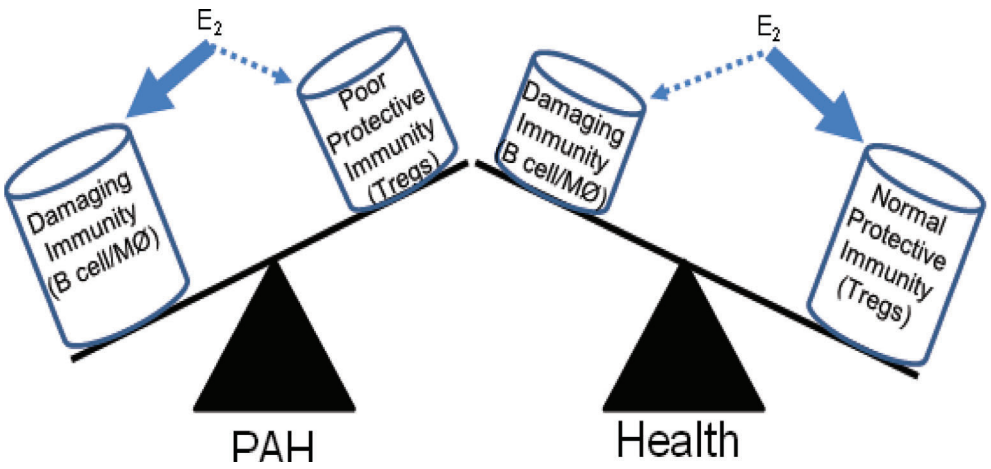

FIGURE 1 | Differential effects of estrogen on immunity in PAH and health. Estrogen (E_2) has complex effects on the immune response. In the absence of normal Treg immunity, E_2 may promote damaging B cell and macrophage responses that induce pulmonary vascular disease and PAH. In health, the positive effects of E_2 fostering Treg maturation may dominate.

significant decline in right ventricular (RV) capillary density, and greater RV perivascular and interstitial fibrosis (22). Treg infusion mitigates PAH and prevents these pathologic changes. Further, prostacyclin (PGI_2), a potent vasodilator, is decreased in females with PAH and may also contribute to the sex-related differences. Following vascular injury and Treg therapy, PGI_2 blood levels increase dramatically. Cyclooxygenase-2 (COX-2) and PGI_2 synthase (PTGIS), critical enzymes for PGI_2 synthesis, are profoundly upregulated in the lungs of Treg-treated rats. Blocking COX-2, heme oxygenase-1 (HO-1), and Programmed Death Ligand-1 (PD-L1)/PD-1 signaling pathways abrogate Treg protection from PAH (22). Thus, in the absence of regular Treg activity, reduced pulmonary COX-2/PTGIS expression and serum PGI_2 levels correlate with worse disease in females. For these reasons, Treg therapy holds promise for PAH patients by helping them restore PGI_2 production in their affected lung tissue. Presently, how Tregs promote PGI_2 synthesis in the major endothelial cell production sites is unknown.

Pulmonary arteries and RV capillaries remodel in evolving PAH (102). Treg infusion into T cell-deficient rats, treated with SU5416, do not develop pulmonary vascular disease and show increased expression of COX-2, PTGIS, HO-1, and PD-L1 in the smooth muscle cell layer of pulmonary arterioles. The Treg-protected RV, by distinction, demonstrates increased expression of these protective molecules in cardiac intimal cells and myocardium (22). Treg therapy may additionally afford protection in PAH by upregulating vascular wall BMPR2 (**Figure 2**). *In vitro*, Tregs cocultured with cardiac endothelial cells increase the expression of COX-2, PTGIS, HO-1, PD-L1, PGI_2, interleukin-10 (IL-10), and estrogen receptors [64-fold for estrogen receptor-alpha (ER-α) and 22-fold for ER-beta (ER-β)]. Collectively, data from this latter study suggest that Tregs protect against RV injury through augmented biosynthesis of ERs, COX-2, HO-1, PGI_2, and IL-10 in cardiac vascular endothelial cells; this activity points to a homeostatic endothelial cell-Treg interaction.

AN EMERGING RATIONALE FOR TREG THERAPY TO TREAT PAH

Treg cell therapy holds treatment promise for a variety of conditions (103, 104). Purified Tregs from the patient's blood are expanded, conditioned, and infused back into the donor's circulation. Prior studies support the rationale for now developing Treg immunotherapy protocols as a treatment for clinical PAH. This effort will benefit from 1) understanding how infused Tregs home to lungs and draining lymph nodes, 2) discerning how Tregs contribute to vascular repair with more established pulmonary vascular disease, and 3) developing strategies to expand Tregs *ex vivo* while enhancing their activities and stability. Relatively small numbers of Tregs are needed to prevent PAH in rats, but their efficacy with these low numbers diminishes if administered after the disease has progressed (21). Existing literature suggests that the infusion of logarithmically increased numbers of Tregs substantially empowers the treatment effect in established disease (105) and could do so for PAH. More than 50 Treg-infusion clinical trials are being tested for autoimmune and inflammatory diseases (103, 104). Co-medication of IL-2, IL-7, or IL-33 promotes Treg survival and proliferation after cell transfer, and strategies that increase *FOXP3* expression improve Treg persistence and suppressive function (103). Increasing Treg specificity is promoted through genetic engineering that enhances chimeric antigen receptors (CAR) expression in Tregs (106). However, to date, there are no consistent antigen targets for PAH, and polyclonal Treg infusions may be the most feasible starting approach.

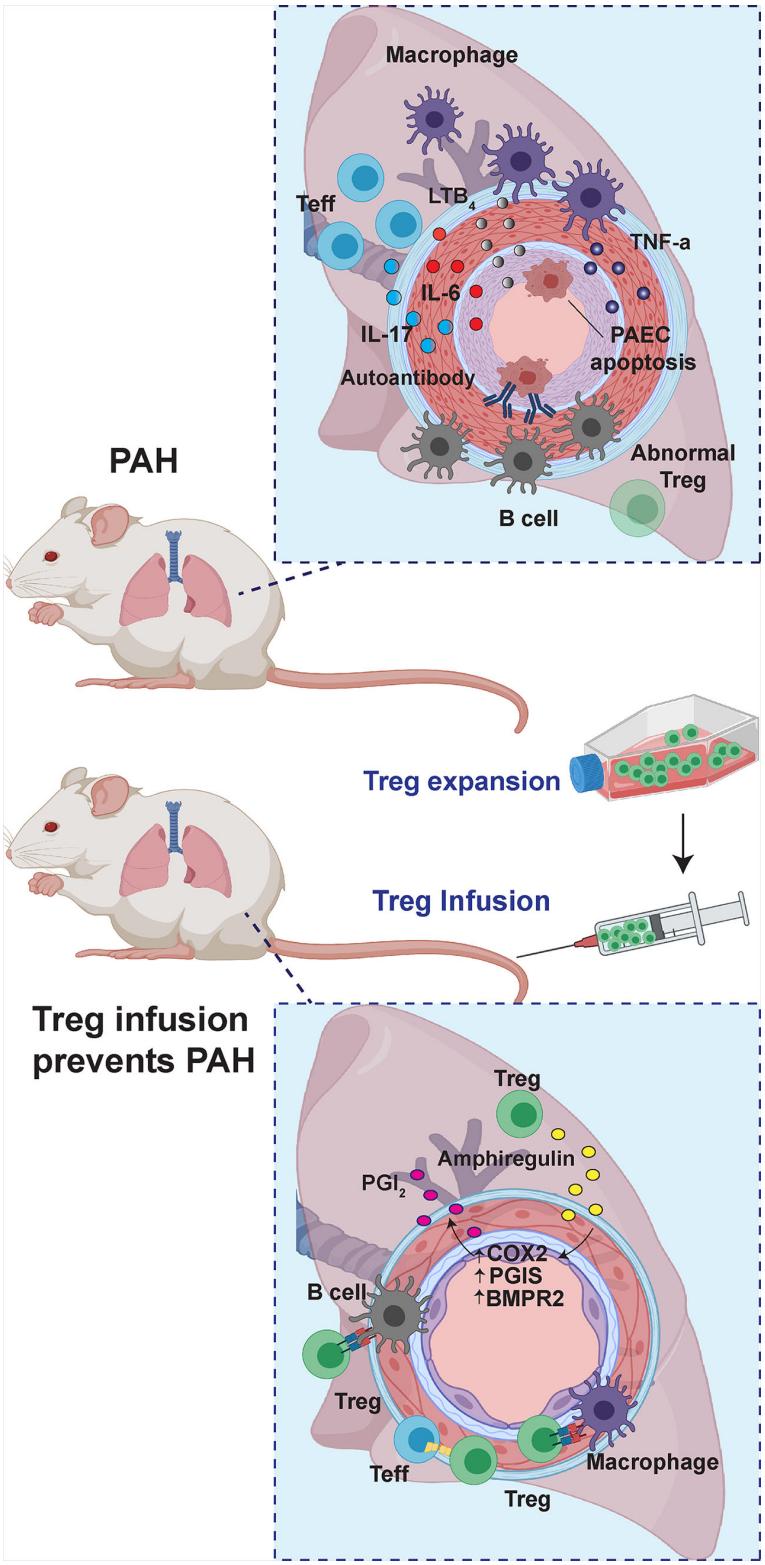

FIGURE 2 | The role of Tregs in PAH. In Treg-deficient rats, vascular injury culminates in enhanced B cell and macrophage-dominated inflammation. With Treg infusion before the induction of vascular injury, PAH is avoided through upregulation of anti-inflammatory and vasoprotective pathways, which include upregulation of COX2, PGIS, and BMPR2.

CONCLUSIONS

Treg dysfunction is a feature of PAH and contributes to immune dysregulation observed in the disease (9, 107). A unique theme of the research presented in this mini-review is that Tregs can directly afford protection to vascular cells, in addition to its better-studied anti-inflammatory effect on other immune cells. In an athymic rat model treated with SU5416 to induce pulmonary vascular injury, Treg infusion also protected right heart function. Sexual differences are present in various PAH manifestations. Consequently, the study of how regulatory immunity differentially impacts men and women with this condition continues to be an area of promising investigation. Combining immune modulators with vasodilators offers potentially better treatment for PAH. Such an approach was recently taken with a randomized multi-center placebo-controlled clinical trial testing B cell depletion with rituximab to treat systemic sclerosis-associated PAH (108); therapy was safe and potentially effective as an adjunct to standard-of-care vasodilators. For this reason, as Treg therapy becomes a therapeutic option for a variety of immunological disorders (109, 110), vulnerable PAH patients represent a new and promising target population.

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All authors (WT, SJ, XJ, RT, DK, TG, SA, SP, NV, QT, and MN) contributed to the design, writing and review of this mini review. All authors contributed to the article and approved the submitted version.

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Antigen Specific Regulatory T Cells in Kidney Transplantation and Other Tolerance Settings

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Kidney transplantation is the most common solid organ transplant and the best current therapy for end-stage kidney failure. However, with standard immunosuppression, most transplants develop chronic dysfunction or fail, much of which is due to chronic immune injury. Tregs are a subset of T cells involved in limiting immune activation and preventing autoimmune disease. These cells offer the potential to provide tolerance or to allow reduction in immunosuppression in kidney transplants. The importance of Tregs in kidney transplantation has been shown in a number of seminal mouse and animal studies, including those with T cell receptors (TCRs) transgenic Tregs (TCR-Tregs) or Chimeric Antigen Receptor (CAR) Tregs (CAR-Tregs) showing that specificity increases the potency of Treg function. Here we outline the animal and human studies and clinical trials directed at using Tregs in kidney transplantation and other tolerance settings and the various modifications to enhance allo-specific Treg function *in vivo* and *in vitro*.

Keywords: kidney, transplantation, Treg, antigen, tolerance

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INTRODUCTION

Kidney transplantation is currently the preferred treatment for patients with end-stage kidney disease (ESKD). The primary limitation of long-term kidney allograft survival has been chronic immune-mediated rejection. This is due to both cellular and humoral pathways as well as innate immune factors and possibly other pathways. Major improvements have occurred over time with improved and more specific immune suppression particularly targeting T effector cells. However, because of the deleterious effects of immunosuppression a major goal has been to achieve immune tolerance to the transplant.

In organ transplantation, there has been a longstanding interest in transferring therapies that create tolerance to the clinic (1). In kidney transplantation, a number of bone marrow-based strategies creating temporary or permanent donor hematopoietic chimerism have reached clinical trials. The first of these was done at Massachusetts General Hospital (MGH), followed closely by Stanford, John Hopkins and North Western (2–6).

Regulatory T cells (Tregs) are a subset of T cells that suppress immune activation and limit autoimmunity in the periphery (7). A number of cell types with immune-regulatory function have been characterized as Tregs, though the one best understood, at present, are the $CD4^+FOXP3^+$

Tregs. These either arise in the thymus (tTregs) or develop from peripheral $CD4^+$ T cells that convert into $CD4^+FOXP3^+$ Tregs as peripherally derived Tregs (pTregs) (8). Previously, it had been thought that peripheral tolerance due either tTregs or pTregs would be insufficient for long standing tolerance. However increasing understanding of Tregs and other regulatory subsets has led to the possibility that transplant tolerance allowing reduction or cessation of immunosuppression could be achieved using regulatory cell subsets (9).

We and others have shown a role for Tregs in 1) protecting against autoimmune kidney disease, 2) maintaining tolerant kidney grafts, 3) preventing human islet allograft and pig xenograft rejection in humanised mouse models, and 4) preventing kidney allograft loss in mouse models of kidney tolerance (10–13). In addition to our studies, there have been 3 bone marrow transplant (BMT) trials reporting the use of Tregs in graft *versus* host disease (GVHD), and there are current human trials using Tregs in autoimmune diabetes at UCSF (14–17). Clinically, polyclonal human Tregs have been shown to be safe to infuse in kidney transplant patients in the multi-centre One Study (18) and the safety of this study suggests that Tregs may be the next frontier of cell therapy (19–21). This has also been achieved at North Western University in kidney transplant recipients with *ex-vivo* expanded Tregs (22). This group has also developed protocols for *ex-vivo* expanded allo-antigen specific Tregs (23).

In this review, we focus primarily on antigen specific Tregs induction in kidney transplantation tolerance and other tolerance studies in animal models and human studies of kidney transplantation and discuss several approaches to generate and expand antigen specific Tregs which can protect against transplant rejection.

REGULATORY T CELLS

Tregs that limit autoimmunity have been studied for a long time. Almost 20 years ago the identification of a $CD4^+CD25^+$ subset as necessary to protect against autoimmunity and limit alloresponse and the subsequent identification of Foxp3 as a master transcription factor identified this regulatory subset (24–27). Further, the separation of these $FOXP3^+$ Tregs into tTregs or pTregs developed in the periphery *in vivo* has helped shape an understanding of their action (28). Tregs can function through multiple pathways to induce tolerance. Tregs are likely to have a number of functional subsets that mirror effector T cells (28). Naïve $CD4^+$ T cells can be induced into $FOXP3^+$ Tregs *in vitro* as iTreg in this review. tTregs with epigenetic changes, provide stability of demethylated FOXP3 especially at the Treg-specific demethylated region (TSDR) site, making them are more resistant to conversion to other CD4 T cell phenotypes (29); pTregs and iTregs without TSDR demethylation, appear at risk of conversion into pathogenic subsets of CD4 T cells, including Th17 (30).Many subsets of effector Tregs express lineage-specific transcription factors in combination with the transcription factor B-lymphocyte-induced maturation protein 1 (BLIMP1) which acts as a repressor. The important role of IL-2 and IL-4 in driving alloantigen specific Tregs in transplantation has recently been demonstrated (31). It has also been shown that acquisition of antigen-specific memory by Tregs is associated with more potent function (32). Further recent data suggests that memory Tregs are more potent than naïve Tregs as shown in antigen-specific models of skin inflammation where response to self-antigen imprints regulatory memory in skin tissues and this has also been shown in rat cardiac transplant models (33). Of interest, antigen-specific $CD4^+$ type 1 regulatory T cells that express IL-10 and/or TGF-β (Tr1) can be induced independently of foxp3 expression, can limit antigen-specific immune responses and may also be important as mediators of transplant tolerance (34, 35). Further identification of memory markers on effector Tregs in transplant settings suggests these may be more potent (36).

DONOR ANTIGEN-SPECIFIC TREGS IN ANIMAL TRANSPLANT MODELS

In transplantation, the key goal is to achieve donor-specific transplant tolerance in which the donor allograft is accepted without long-term immunosuppressive requirements, and the recipient remains capable of responding to pathogenic microorganisms and other alloantigens (37). Donor-specific tolerance of allografts arises spontaneously in various animal models (11, 38–40) and has been well-documented in various transplant populations (**Figure 1**), including our study in a pediatric liver transplant recipient with full hematopoietic chimerism (41). As well as Treg therapy, several approaches have been used to achieve transplant tolerance in animal models, including costimulatory blockade, hematopoietic donor chimerism using BMT and stem cell transplantation, targeting of effector cell activation and recruitment, and donor-specific transfusions (DST). Among these approaches, donor-antigen specific $Foxp3^+$ Tregs have been found to play the crucial role in the maintenance of allograft tolerance in several transplant animal models.

Donor Antigen-Specific Tregs in Spontaneous Acceptance of Kidney Allografts

Donor-specific tolerance of renal allografts occurring spontaneously across certain MHC mismatched mouse strains is a valuable model for studying transplant tolerance (11, 38–40). Studies showed spontaneous kidney allografts tolerance occurred in the DBA/2 ($H2^d$) to C57BL/6 ($H2^b$) transplant model and was associated with T cell mediated immune regulation requiring TGF-β (40). In a spontaneous kidney allograft tolerance mouse model with C57BL/6 mice as donors and B10.BR ($H2^k$) as recipients, we found $Foxp3^+$ Tregs increased in tolerant kidney allografts (39). Further we and others demonstrated that $Foxp3^+$ Tregs were essential for tolerance induction in spontaneously accepted kidney allograft from DBA/2 to C57BL/6 mice, where depletion of $CD4^+CD25^+$ Tregs or $Foxp3^+$ Tregs abolished kidney allograft tolerance (11, 42). Using DEREG mice (C57BL/6) that carry the diphtheria toxin receptor and

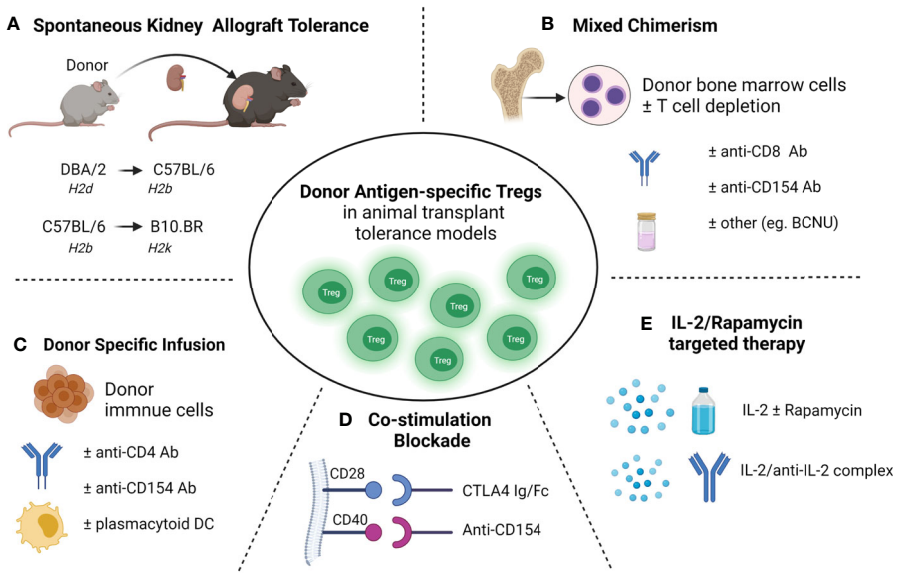

FIGURE 1 | Donor antigen-specific Tregs developed in transplant tolerance mouse models **(A)** Spontaneous acceptance of kidney allotransplant tolerance. **(B)** Mixed chimerism induced allo-transplantation tolerance *via* bone marrow transplantation. **(C)** Donor-specific transfusions (DST) induced allotransplantation tolerance. **(D)** Blockade of the B7-CD28 (using CTLA-4 Ig/Fc) or/and CD40-CD40L (using anti-CD154 mAb) co-stimulatory pathway induced transplantation tolerance; **(E)** and IL-2 and rapamycin targeted therapies induced transplantation tolerance.

enhanced green fluorescent protein (DTR-eGFP) transgene under control of the Foxp3 promoter, we identified donor -antigen-specific Foxp3$^+$ Tregs that were developed in DBA/2 kidney allografts in C57BL/6 recipient mice and confirmed dominant and donor antigen specificity of kidney allograft Tregs *in vivo* in Rag-/- mice (11). These donor antigen-specific Foxp3$^+$ Tregs expressed elevated levels of TGF-β, IL-10, interferon gamma (IFN-γ), BLIMP1 and the chemokine receptor 3 (Cxcr3) (11). These studies demonstrated the crucial role of Foxp3$^+$ Tregs in transplant tolerance and the therapeutic potential of donor antigen-specific Tregs in clinical settings to improve transplant outcomes.

Donor Antigen-Specific Tregs in Allo-Transplantation With DST

In transplantation, Tregs have been shown to develop in the recipients after DST and the critical role of Tregs has been reported in DST models with mismatch at both major histocompatibility class (MHC) class I and class II (43–48). However, other studies reported equal expansion of recipient transplant-specific Tregs in the rejecting and tolerant heart allografts induced through DST combined with anti-CD154 mAb, and without Treg expansion in the spleen (49). Although DST combined with cyclophosphamide treatment failed to prolong BALB/c (H2^d) islet allograft survival in C57BL/6 (H2^b) host mice, additional infusion of either donor-reactive Tregs (5×10^6) or polyclonal Tregs (25×10^6) led to prolonged survival of allogeneic islets in > 70% of C57BL/6 host demonstrating the critical role of Tregs in DST for induction of tolerance (46). Other studies have also reported DST combined with anti-CD154 mAb and plasmacytoid dendritic cells (pDCs) induced BALB/c heart allograft tolerance in C57BL/6 hosts in which Tregs developed and expanded in the lymph node (47). In a MHC-mismatched skin allotransplant mouse model, mice pre-treated with anti-CD4 monoclonal antibody (mAb) and DST achieved dominant tolerance which was mediated by Tregs in a CTLA-4 and IL-10-dependent manner. However CD4$^+$CD25$^+$ Tregs from graft tolerant mice had no demonstrable specificity for the tolerizing donor antigens (50). In models using DST with additional immunological manipulation, expanded alloreactive Tregs occurred predominantly through the direct pathway of allorecognition (51) and long-term graft acceptance predominantly relied on indirectly activated Tregs (45, 52). Interestingly, in a single MHC-I mismatch skin transplant model, we demonstrated dominant F1 skin-allograft [C57BL/6.C (H-2^{bm1}) × C57BL/6 (H2^b)] tolerance induced by a single DST across an unmanipulated C57BL/6 host (53). In this model, expansion of antigen-specific Foxp3$^+$ Tregs in F1 grafts and spleens of recipient mice were due to thymus-derived Foxp3$^+$ Treg proliferation that were indirectly activated. The essential role of Foxp3$^+$ Tregs in this model was confirmed by depletion of Foxp3$^+$ Tregs in DEREG mice which abrogated F1 skin graft tolerance (53).

Donor Antigen-Specific Tolerance in Allo-Transplantation Combined With BMT

Kidney transplant tolerance in a clinical setting has been achieved with non-myeloablative BMT to induce hematopoietic mixed chimerism, but is limited by the risk of GVHD, and loss of both mixed chimerism and tolerance (3, 54, 55). Mixed chimerism achieves transplant tolerance through both central and peripheral depletion of alloreactive cells (54). More recent data support an

important role for Tregs in mixed chimerism, both in limiting GVHD and maintaining chimerism with host Treg cells involved in preventing chronic allograft rejection and donor Treg cells controlling GVHD in animal models and transplant recipients (56–61). Mouse studies have shown an important role for host Treg cells in tolerance using nonmyeloablative BMT, costimulatory blockade and total body irradiation to achieve mixed chimerism across a full MHC mismatch (58). Mixed chimerism only occurred in the presence of host Tregs in host mice and allogeneic T cell-depleted BMT in a non-lymphoablative irradiation regimen (57). Moreover, host Tregs specific for directly presented donor antigens prevented acute rejection only of skin and heart allografts, while host Tregs specific for both directly and indirectly presented alloantigens prevented both acute and chronic rejection in the same models (57). Using a non irradiation regimen in which the host received, anti-CD154 mAb, a single dose of depleting CD8 mAb, N,N-bis(2-chloroethyl)-nitrosourea (BCNU, an alkylating agent), and allogeneic BMT from methyl-guanine-methyl-transferase (MGMT) transgenic mice, high levels of donor mixed chimerism without GVHD were achieved across fully MHC mismatched mice leading to donor-specific skin allograft tolerance (56). MGMT is a DNA repair enzyme that removes BCNU from guanine and limits BCNU toxicity, enhancing engraftment of donor hematopoietic cells. Importantly, both donor and host $Foxp3^+$ Tregs were expanded in this model, suggesting their important role for the maintenance of tolerance in mixed chimerism (56). In non-human primates (NHPs) where kidney allograft tolerance was achieved with the development of transient mixed chimerism using T cell-depleted BMT and costimulatory blockade without myeloablation, $Foxp3^+$ Tregs were found to be enhanced in tolerant recipients (62). Thus, mixed chimerism and Tregs are likely to be synergistic in achieving transplant tolerance and limiting GVHD in clinical transplantation (63–65).

Donor Antigen-Specific Tregs in Costimulation Blockade–Induced Transplant Tolerance

In several transplant model systems, blockade of the B7-CD28 (using CTLA-4 Ig/Fc) or CD40-CD40L (using anti-CD154 mAb) co-stimulatory pathway induced donor graft tolerance (66–72). The impact of Treg numbers and functionality on the efficacy of costimulation blockade, and the requirement of Tregs in transplant induction *via* co-stimulation blockade is an area of active investigation (67, 68, 73, 74). Although CTLA-4–Ig treatment in C57BL/6 naïve mice causes a decrease in Treg and $Helios^+Foxp3^+$ Tregs, it prolongs fully mismatched BALB/c heart allograft survival in C57BL/6 recipients. However, it does not protect single MHC-II mismatched heart allografts in C57BL/6 recipients in which long-term allograft survival was Tregs dependent (73). In a different model, treatment with CTLA4-Ig abolished skin allograft tolerance induced by IL-2/anti–IL-2 complex suggesting that allo-Treg development requires costimulation (75). Others reported that in the presence of B7:CD28 and CD40:CD40L co-stimulatory blockade, the suppressive function of $CD4^+CD25^+$ Tregs was activated, suppressing the proliferation of $CD4^+$ effector cells (67). Experiments *in vitro* by Vogel et al. found that co-stimulatory blockade primed $Foxp3^+$ Tregs to be more suppressive than naïve $Foxp3^+$ Tregs (76). Depletion of $CD4^+CD25^+$ Tregs in recipient mice abolished heart allograft tolerance induced by CD154 mAb treatment (77). In an islet xenotransplant pig-mouse model, we found expanded $Foxp3^+$ Tregs populations in the xenograft, spleen and draining lymph nodes that were induced by treatment with CTLA-4 Fc and anti-CD154 mAb. Once again activated $Foxp3^+$ Tregs had more potent regulatory function *in vivo* than naive Tregs (68). Further, depletion of $Foxp3^+$ Tregs at induction (unpublished) or maintenance (68) in recipient mice with the blockade of B7-CD28 or CD40-CD40L abolished transplant tolerance thereby confirming the essential role of these cells. However, antigen specificity of Treg after the induction of tolerance does not appear to be universal. Using a combination of nondepleting CD4, CD8, and CD154 mAb to induce fully mismatched C57BL/10 (B10, $H\text{-}2^b$) skin allograft tolerance in CBA ($H2^k$) mice, antigen specific tolerance to the second skin allograft was shown to be mediated by $CD4^+CD25^+$ Tregs; but these Tregs from tolerant mice had no demonstrable donor antigen specificity after adoptive transfer (78).

Donor Antigen-Specific Tregs in IL-2 and Rapamycin Targeted Therapies

The cytokine interleukin-2 (IL2) is a key regulator of immune cells including Tregs that constitutively express the high-affinity IL-2Rα (CD25) and are highly sensitive to very low doses of IL-2 (79–81). In the clinic, low-dose IL-2 therapy has been shown to selectively expand FOXP3 Tregs and used successfully to treat chronic GVHD (82), hepatitis C virus–induced vasculitis (83), systemic lupus erythematosus (SLE) (84), and type 1 diabetes (85, 86). In a humanized mouse model, we demonstrated that the infusion of *in vitro*–expanded human $FOXP3^+$ Tregs in the presence of IL-2 and rapamycin prevented porcine neonatal islet cell cluster xenograft rejection (87). Low dose IL-2 treatment in the host mice led to expansion of $Foxp3^+$ Tregs *in vivo* and prolonged allograft survival in a full MHC-mismatch corneal transplant model of C57BL/6 grafts to BALB/c hosts, and these *in vivo* expanded Tregs also demonstrated donor antigen specificity (88). Further, IL-2 treatment of host mice preferentially expanded infused donor antigen-specific Tregs generated *in vitro*, and improved the skin allograft survival in a single MHC-mismatch mouse model (89). Using IL-2/anti-IL-2 antibody complexes has also been shown to induce $Foxp3^+$ Tregs expansion, prolong islet allograft survival (90) and protect against proteinuric chronic kidney disease (91). In our study, low dose of IL-2 treatment alone failed to prolong islet allograft survival in humanized mice despite expanding$FOXP3^+$ Tregs *in vivo* (92). Graft survival depended on the balance of effector T cells and Tregs. Rapamycin has been shown to be pro-tolerogenic by allowing expansion of human and mouse Tregs while also suppressing $CD4^+$ T effector cells in experimental studies (93–96). Whilst low-dose rapamycin treatment did not lead to either Treg expansion *in vivo* or prolongation of islet graft survival in humanized mice, the combination of IL-2 and rapamycin led to an expansion of Tregs and prolonged human islet allograft survival in this humanized mice model (92). The combination of IL-2 and rapamycin has also

been reported to prolong skin graft survival in minor antigen mismatch and semi-allogeneic mouse recipients (97). These studies suggest a combination of agents that selectively expand Tregs *in vivo* whilst limiting effector T-cell expansion will be required to protect graft rejection if these therapies are to be translated in the clinic. Although these results in rodent studies are encouraging, more work needs to be done to ensure that T cell activation *in vivo* is limited to the Treg population only. IL-2, at the appropriate dose, can activate effector T cells, and a clinical trial of low dose IL-2 in liver transplant recipients had to be ceased due to increased rejection without evidence of Treg induced suppression (NCT02949492).

POTENTIAL METHODS TO GENERATE AND INDUCE ALLOSPECIFIC TREGS *EX VIVO*

Concerns around non-specific immune suppression and the need to increase potency has led to the development of antigen-specific Tregs which display more efficient suppressive function than naïve Tregs. There are several methods to generate donor antigen-specific Tregs from FOXP3$^+$ Tregs (CD4$^+$CD25$^+$CD127$^-$) precursors, and antigen-specific iTregs or Tr1 from naïve CD4$^+$ T cells for therapy in transplantation (**Figure 2**) (98, 99).

Direct Antigen Expansion With Donor HLA Expressed by APC

Human Tregs expanded in the presence of donor antigen *ex vivo* demonstrate enhanced suppressive function and were more effective for protecting skin allograft rejection in a humanized mouse model (100). Our group has identified xenospecific human CD27$^+$HLADR$^+$ Tregs generated in the presence of pig donor peripheral blood mononuclear cells (PBMCs) in a mixed lymphocyte reactions (MLR) system which demonstrated more effective protection of pig islet xenograft rejection in a humanized mouse model (unpublished). Allospecific Tregs were generated using isolated CD4$^+$CD25$^+$CD127$^-$ Tregs from PBMCs of healthy volunteers in the presence of soluble 4-trimer CD40 ligand (CD40L)-activated donor B cells (23) or skin-derived myeloid DCs (101). Further studies have shown that human allospecific Tregs can be generated in a MLR system from PBMC of uremic patients in the presence of donor PBMCs under conditions of costimulatory blockade with belatacept (102). Allospecific Tregs were generated in different MLR system in which sorted CD4$^+$CD25$^+$CD127$^-$ Tregs from transplant recipient' PMBCs were expanded with donor B cells activated using K562 cells and expressing human CD40L (100). These allospecific Tregs maintained high levels of FOXP3 expression and TSDR (100, 102), and demonstrated safety for cell therapy in clinical kidney transplant trials (18, 100, 102).

Indirect Antigen Expansion With Donor HLA Fragments Presented by Host APC/MHC

Another approach has been the use of indirectly presented HLA molecules in the context of recipient MHC using antigen presenting cells (dendritic cells or B cells) pulsed with donor antigen. This may potentially be more effective for suppression of chronic rejection (103). However, this is often limited by the small clone size of TCR recognizing the peptide in the context of

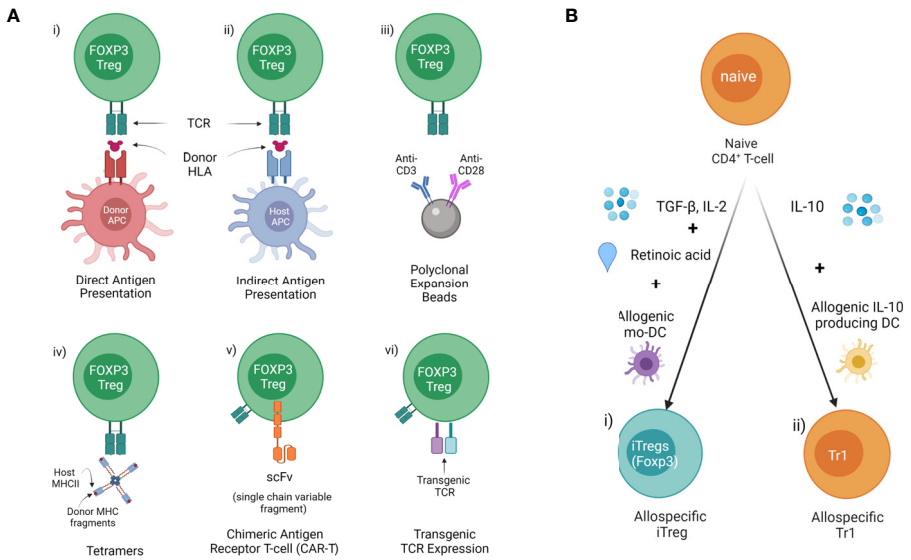

FIGURE 2 | Methods of allospecific Treg derivation **(A)** Generating donor antigen-specific Tregs from FOXP3$^+$ Tregs (CD4$^+$CD25$^+$CD127$^-$) precursors. This includes donor HLA presented to generate i) direct antigen expanded (donor APC/MHCII) or ii) indirect antigen expanded Tregs ed (host APC/MHCII). iii) Polyclonal expansion with antiCD3/CD28 beads. iv) Isolation of specific Tregs using Tetramers composed of host class II MHC & donor MHC fragments. v) Insertion of antibody fragment scFv connected to TCR to create a CAR. vi) Genetic modification of Tregs with transgenic TCR. **(B)** Inducing donor antigen-specific i) iTreg and ii) Tr1 from naïve CD4$^+$ T cells.

self-MHC or the development of inhibitory constimulatory molecules by the APC.

Isolation of Specific Tregs Using Tetramers Composed of Host Class II MHC & Donor MHC Fragments

This has the advantage of having a broad range of T cell receptors (TCRs) in the selected population. It still requires Treg expansion and sorting. It also has issues around the initial selection of Tregs and it may potentially be useful in displaying a range of targets. However MHC Class II tetramers have proved less efficient than Class I and may be a barrier to larger scale clinical use (104).

Insertion of Allospecific TCR

The use of TCRs against an alloantigen was first used by Simpson and her group using TCR transgenic mice against the H-Y antigen (105, 106). Here Tregs directed against H-Y could induce tolerance against a minor antigen (107, 108). TCR-transduced Tregs specific for allogeneic MHC class II molecules induced long-term survival of partially MHC-mismatched heart grafts when combined with short-term adjunctive immunosuppression (109). This demonstrated the capacity and increased potency of TCR targeted Treg therapy. More recently this approach has been used in cancer against minor H-A antigens but may have a future role in transplantation tolerance (110).

Car-Tregs

Antibody fragment scFv connected to the intracytoplasmic tail of the TCR and associated signaling molecules are transduced into Tregs to create a chimeric antigen receptor (CAR) Treg (111). The targeting of Tregs in organ transplantation and in autoimmunity to the transplanted cells or autoimmune tissues is an attractive option. The potency of antigen targeting was first shown by the Simpson group as described above. T cell specificity has also been achieved using antibody fragments linked to intracellular TCR signalling domains called CARs. CAR T cells have been used most impressively in the treatment of CD19 lymphoma using CD8 T cells engineered with a scFv chain from an antibody against CD19 fused to the intracellular component of the T cell receptor (112–114). These hybrid receptors are comprised of a scFv derived from monoclonal antibodies or an antigen-binding fragment (Fab) fused to CD3-ζ transmembrane and end domains, the intracellular functional component of the TCR. This approach has been used to direct T cells against cancer antigens, most spectacularly against CD19. Further modifications enhance the survival and activity of the Treg and drive the functionality of the T cells have been undertaken (115).

The efficacy of CAR Tregs directed against alloantigens has been demonstrated using HLA-A2 specific CAR T cells *in vitro* and in transplant models (116–118). However, CAR Tregs while limiting skin graft rejection in naïve mice cannot prevent skin graft rejection in sensitized mice (119). Many strategies exist to improve the isolation and expansion of Tregs and subsequent genetic modifications to alter cytokine signalling, enhance regulatory elements or inhibit signals that supress regulation and develop pathways for testing in large animals and human trials (120).

Generating Allospecific iTregs From Naïve $CD4^+$ T Cells

iTregs are induced to express FOXP3 from naïve $CD4^+$ under specific condition *in vitro*, but iTregs without TSDR demethylation, appear more susceptible to conversion into pathogenic subsets of $CD4^+$ T cells (30). iTregs induced with TGF-β in the presence of all-trans retinoic acid (ATRA) and rapamycin demonstrated robust suppressive function *in vitro* but not *in vivo*, in the humanized GVHD mouse model (121). Interestingly, allospecific iTregs that were induced with TGF-β1, IL-2, and ATRA in the presence of allogeneic monocyte-derived dendritic cells, can specifically suppress donor allo-responses but not third-party allo-responses, and maintain suppressive function in the presence of pro-inflammatory cytokines, despite methylation of the FOXP3 TSDR (122).

Generating Allospecific Tr1

Tr1 cell therapy in GVHD trials including NCT03198234 suggested therapeutic possibility in the treatment of transplantation rejection (123). Allospecific Tr1 were enriched in cultured $CD4^+$ T cells that were stimulated with allogeneic IL-10-producing DCs generated from $CD14^+$ monocytes in the presence of IL-10 (124). These allospecific Tr1 showed specific suppression function to donor alloresponse *in vitro* and maintained a tolerogenic gene expression profile *in vivo* (124).

ANTIGEN-SPECIFIC TREGS IN TRANSPLANT CLINICAL TRIALS

Given the central role of T-cells in downstream effector function, Tregs have been studied as a potential therapeutic in human solid organ transplantation. Sixteen Treg clinical trials that have focused on feasibility, safety and preliminary efficacy of infused Tregs to reduce the dose of calcineurin inhibitor (CNI)-based immunosuppression have been reported. However, the majority of reported clinical trials using Tregs lack the capacity to robustly identify mechanistic effects and much of our understanding of underlying mechanisms relies on extrapolation of results from rodent and non-human primate studies.

Autologous or donor-derived peripheral blood is the most common source of Tregs used in clinical trials, although alternate sources such as umbilical cord blood and paediatric thymus, non-lymphoid tissue such as skin, adipose tissue and muscle have been explored (125) (**Figure 3**). Peripheral blood contains only a small proportion of Tregs that require purification *via* $CD25^+$ cell selection, with or without $CD8^+$ depletion. *Ex vivo* expansion of these naturally-occurring Tregs can be achieved through various means, but the choices are dependent on whether polyclonal or donor alloAg-reactive Treg (darTreg) are required (100). darTregs are theoretically more potent, however the majority of trials have expanded polyclonal Tregs with a combination of IL-2 and anti-CD3/CD28 magnetic bead stimulation ± rapamycin or TGF-β, although this process may require a greater cell infusion dose to achieve donor-specific suppressive effects (126–129) (**Figure 3**).

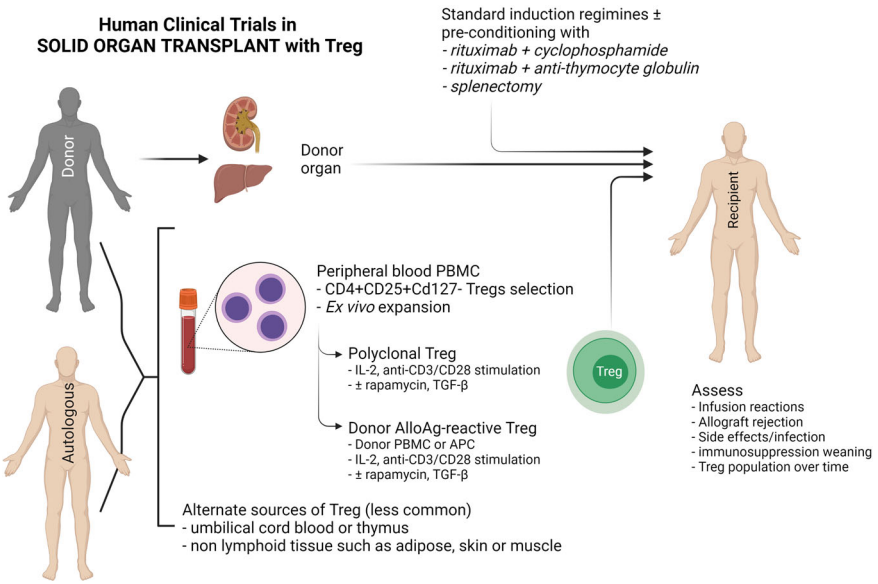

FIGURE 3 | Schematic diagram of current clinical Treg trials in kidney and liver transplantation. This diagram indicates the sources of FOXP3$^+$ Tregs ($CD4^+CD25^+CD127^-$), the two main methods of Treg expansion and assessment in clinical trials.

Clinical studies in kidney or liver transplantation have demonstrated Treg safety in early phase I/II studies. The One study (18), which included polyclonal Treg (n = 12 and 11 at Guy's and Oxford University Hospitals, UK and Charité Hospital, Germany) and darTregs (n = 2) at the University of California San Francisco Medical Centre and (n = 3) at Massachusetts General Hospital, USA, did not demonstrate significant adverse events such as rejection. However, infusion of darTregs following a preconditioning regimen of splenectomy, rituximab with cyclophosphamide, or rituximab with rabbit anti-thymocyte globulin in living donor kidney transplantation resulted in significant rejection risk following immunosuppressive drug weaning (130). This highlights the caution required to achieve *in vivo* translation of cell therapies – despite demonstrating functionally suppressive Tregs *in vitro*, there was either insufficient Treg dosage, loss of regulatory function *in vivo*, or significant depletion of recipient Tregs. Additional studies of darTregs in liver transplantation (deLTa, NCT02188719) was not completed within the study timeframe, and several additional studies (ThRIL, NCT02166177; ARTEMIS NCT02474199) are either not formally reported or yet to commence recruiting (LITTMUS, NCT03654040). Tr1 cell therapy in two kidney transplant patients in the One Study group showed Tr1 cells expand and Tr1-cell tolerogenic gene expression profile remains stable even under active immunosuppressive treatment (124).

CONCLUSIONS

The safety of infusing mildly manipulated Tregs has been demonstrated in clinical trials of kidney transplantation. Promising animal studies suggest that specific Tregs or modified Tregs may be more potent and allow further reduction in immunosuppression or true tolerance. There are caveats with CAR T cells failing to suppress rejection in sensitized mice and primate studies demonstrating Treg conversion. However overall, the accumulating evidence suggests that these barriers can be overcome and allo-specific Tregs therapy translated into the clinical practice.

AUTHOR CONTRIBUTIONS

MH drafted the sections of donor antigen specific Tregs in animal transplant models, revised and edited the paper. NR drafted the section of antigen specific Tregs in transplantation clinical trials, revised and edited the paper. JL drafted the figure and edited the the paper. GZ, YW, and KS revised the draft. PO'C revised the draft and edited the paper. SA drafted the sections of introduction, potential methods to induce allospecific Tregs *ex vivo*, and conclusions, edited and finalized the paper. All authors contributed to the article and approved the submitted version.

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Precision Engineering of an Anti-HLA-A2 Chimeric Antigen Receptor in Regulatory T Cells for Transplant Immune Tolerance

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Infusion of regulatory T cells (Tregs) engineered with a chimeric antigen receptor (CAR) targeting donor-derived human leukocyte antigen (HLA) is a promising strategy to promote transplant tolerance. Here, we describe an anti-HLA-A2 CAR (A2-CAR) generated by grafting the complementarity-determining regions (CDRs) of a human monoclonal anti-HLA-A2 antibody into the framework regions of the Herceptin 4D5 single-chain variable fragment and fusing it with a CD28-ζ signaling domain. The CDR-grafted A2-CAR maintained the specificity of the original antibody. We then generated HLA-A2 mono-specific human CAR Tregs either by deleting the endogenous T-cell receptor (TCR) *via* CRISPR/Cas9 and introducing the A2-CAR using lentiviral transduction or by directly integrating the CAR construct into the TCR alpha constant locus using homology-directed repair. These A2-CAR^{+}TCR^{deficient} human Tregs maintained both Treg phenotype and function *in vitro*. Moreover, they selectively accumulated in HLA-A2-expressing islets transplanted from either HLA-A2 transgenic mice or deceased human donors. A2-CAR^{+}TCR^{deficient} Tregs did not impair the function of these HLA-A2^{+} islets, whereas similarly engineered A2-CAR^{+}TCR^{deficient}CD4^{+} conventional T cells rejected the islets in less than 2 weeks. A2-CAR^{+}TCR^{deficient} Tregs delayed graft-*versus*-host disease only in the presence of HLA-A2, expressed either by co-transferred peripheral blood mononuclear cells or by the recipient mice. Altogether, we demonstrate that genome-engineered mono-antigen-specific A2-CAR Tregs localize to HLA-A2-expressing grafts and exhibit antigen-dependent *in vivo* suppression, independent of TCR expression. These approaches may be applied towards developing precision Treg cell therapies for transplant tolerance.

Keywords: chimeric antigen receptor, regulatory T cells, genome editing, transplantation, humanized mouse model, immune tolerance, HLA, Treg

INTRODUCTION

Regulatory T cells (Tregs) are a small subset of $CD4^+$ T cells that are key for maintaining self-tolerance and preventing autoimmune disease (1). A plethora of preclinical models have shown that the infusion of Tregs can suppress graft rejection and promote transplant tolerance (2). Several phase I/II clinical studies using Tregs have been reported (3, 4). For instance, the ONE Study is the largest coordinated international study of regulatory cell therapies in kidney transplantation. The study includes 28 patients who received Treg therapy in 4 non-randomized single-arm phase I/IIa trials. The results demonstrated feasibility, safety, and potential benefit of Treg-based therapies to reduce the burden of immunosuppression (5). While a significant fraction of Tregs in the polyclonal pool can react to allogeneic donor antigens, data from preclinical models show that donor-reactive Tregs are more effective than polyclonal Tregs in promoting transplant tolerance (6). Unfortunately, donor alloantigen-reactive Tregs may be functionally altered or induced to migrate out of the peripheral blood following transplantation, thus limiting the frequency of alloantigen-reactive clones within polyclonal Treg products and thereby posing challenges for consistent expansion of donor-reactive Tregs (2).

Redirecting Tregs with transgenic T cell receptors or chimeric antigen receptors (CAR) have been reported by multiple labs since 2005 (7–14). In transplantation, engineering alloantigen reactivity using an alloantigen-specific CAR has also been reported (15–17). Previous studies have shown that a CAR, consisting of a mouse anti-HLA-A2 (A2) antibody-derived single chain variable fragment (scFv) coupled to a CD28-ζ signaling domain, could be introduced in human Tregs using lentivirus. These A2-CAR Tregs demonstrated superior efficacy in preventing xenogeneic graft-*versus*-host disease (GvHD) in NSG mice when compared to polyclonal Tregs or Tregs transduced with an irrelevant CAR (18). The therapeutic potential of A2-CAR Tregs for organ transplantation was subsequently demonstrated by two separate groups which independently applied A2-CAR Tregs to prevent the rejection of $A2^+$ human skin grafts in humanized mouse models, further bolstering the enthusiasm for evaluating this technology in humans (19, 20).

In all these studies, the CAR constructs were introduced into the Tregs *via* lentivirus and the engineered Tregs also expressed their endogenous TCR. Lentiviral transduction results in random integration of the CAR construct in the genome that can lead to variable levels of CAR expression, transcriptional silencing, or accidental disruption of important genes. A previous study has shown that site-specific integration of a CD19-CAR into the TCR alpha constant region (*TRAC*) of T cells results in a more uniform distribution and TCR-like regulation of CAR surface expression, thereby mitigating T-cell exhaustion and enhancing anti-tumor activity (21). In addition, we recently observed that CAR^{hi} human T effector cells exhibited a surprisingly robust proliferative response to anti-CD28 stimulation alone, independent of CAR or TCR engagement, whereas CAR^{lo} T effector cells did not (22). Thus, lentivirally engineered Tregs may result in heterogeneous CAR expression and unexpected properties of the engineered cells. Knocking a CAR into the *TRAC* locus and deleting the endogenous TCR may more precisely control CAR Treg activity. Furthermore, this strategy avoids confounding effects from xenoreactivity of the endogenous human TCR against mouse antigens when testing the *in vivo* function of CAR Tregs in humanized mouse models. However, it is unclear whether CAR Tregs can function without the endogenous TCR. We thus conducted the current study by generating $TCR^{deficient}$ A2-CAR human Tregs and assessed their trafficking, survival, and function in humanized NSG mouse hosts.

MATERIALS AND METHODS

Human Peripheral Blood Products and T Cell Isolation And Expansion

Human peripheral blood from de-identified healthy donors was purchased from STEMCELL Technologies (Vancouver, Canada), which collects and distributes de-identified human blood products with consent forms, according to protocols approved by the Institutional Review Board (IRB). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (GE Healthcare, Chicago, IL) density gradient centrifugation. T cells were further enriched using the EasySep Human T Cell Isolation Kit (STEMCELL Technologies), as per the manufacturer's instructions. Enriched $CD3^+$ T cells, or $CD4^+CD127^+CD25^{low}$ conventional T cells (Tconv) or $CD4^+CD127^{low}CD25^{high}$ regulatory T cells (Tregs) purified by fluorescence-assisted cell sorting (FACS) using a BD FACS Aria II Cell Sorter (Beckton Dickinson, Franklin Lakes, NJ) were used for experiments. Tregs were expanded as previously described (23). Antibodies utilized for flow cytometry are summarized in **Supplementary Table 1**.

Cloning and Specificity Verification of an Anti-HLA-A2 scFv

A human B-cell derived hybridoma (clone SN607D8) was used as source material to produce an anti-HLA-A2 scFv. This hybridoma produces an IgG1κ monoclonal antibody that recognizes HLA serotypes A2 and A28 (24). RNA from the SN607D8 hybridoma was used as template for RT-PCR amplification of the V_L and V_H chains of the IgG. The scFv gene was then constructed in a V_H-$(GGGS)_3$linker-V_L format and incorporated into the pHEN1 phage display vector (25). The binding activity of phage-displayed scFv was assessed using two tumor cell lines, THP-1 [HLA-A*02:01/02:01, HLA-B*15:11/15:11 (26)] and RPMI 8226 [HLA-A*30:01/68:02, HLA-B*15:03/15:10 (27)]. Binding to these cell lines was measured using sequential staining with a biotinylated anti-phage antibody and fluorochrome-conjugated streptavidin followed by flow cytometric analysis.

Grafting of the Anti-HLA-A2 scFv

The CDR regions of the anti-HLA-A2 scFv from hybridoma SN607D8 were grafted onto the 4D5 human antibody scaffold

used in herceptin (trastuzumab) by pairwise alignment of amino acid residues using the software Jalview (28). The specific CDR3 regions of the anti-HLA-A2 scFv were predicted using the software Paratome (29). The grafted scFv was constructed in the V_H-$(GGGGS)_3$linker-V_L format.

Lentivirus Production

The A2-specific CAR was created by generating a chimeric DNA sequence encoding a MYC-tag upstream of the grafted anti-HLA-A2 scFv, an IgG4 hinge, CD28 transmembrane domain, and a CD28-CD3zeta tandem signaling domain (purchased as gblocks from Integrated DNA Technologies, IDT, Coralville, IA). The resulting DNA fragment was subcloned into a pCDH lentiviral vector containing an EF1α promoter [addgene-plasmid-64874 (30)]. The CAR construct was linked to a truncated EGFR (EGFRt) or a luciferase gene *via* a 2A self-cleaving peptide sequence. All constructs used in subsequent experiments were confirmed by Sanger sequencing. Lentivirus was produced as previously described (31). Briefly, HEK293T cells were seeded at 3×10^6 cells on 10 cm cell culture dishes 24 hours prior to transfection with 4 μg of plasmid DNA, 2 μg of the packaging vector pCMV-dR8.9, 2 μg of VSV envelope vector pMD2.G and 15 nmol linear 25 kDa polyethylenimine (Millipore Sigma, Burlington, MA). Media was replaced 24 hours later and ViralBoost Reagent (Alstem, Richmond, CA) was added. The supernatant was collected 24 and 48 hours later. Virus was concentrated using LentiX concentrator (Takara, Shiga, Japan).

AAV6 Production

A pAAV-MCS plasmid containing inverted terminal repeats (ITRs) from AAV serotype 2 (Agilent Technologies, Santa Clara, CA) was utilized as backbone for AAV6 plasmid construction [naturally occurring AAV6 has an AAV2 ITR (32)]. Cloning was performed with in-fusion cloning tools and protocols provided by Takara. Large scale DNA preparation was performed using a Zymopure plasmid maxiprep kit (Zymo Research, Irvine, CA). All constructs used in subsequent experiments were confirmed by Sanger sequencing. For AAV production, 30 μg of pDGM6 helper plasmid (a gift from Dr. YY Chen, University of California, Los Angeles), 40 μg of pAAV helper containing the VA, E2A and E4 regions (33), a gift from Dr. YY Chen, University of California, Los Angeles), and 15 nmol linear polyethylenimine were used. AAV6 vector production was carried out by iodixanol gradient purification as previously described (34, 35). After ultracentrifugation, AAVs were extracted by puncture and further concentrated using a 50 ml Amicon column (Millipore Sigma) and titrated directly on primary human T cells.

HLA Allele Cross-Reactivity Assay

HLA allele cross-reactivity of the A2-CAR-expressing Tregs was determined based on a previously reported method (36). In brief, 2.5×10^4 FACS-purified A2-CAR Tregs, as well as 2.5×10^4 control untransduced polyclonal Tregs, were incubated with 0.5 μl to 5 μl PE-labeled FlowPRA Single HLA Antigen bead panels (FL1HD01 and FL1HD02, OneLambda, Los Angeles, CA), a fixable viability dye (Ghost Dye BV510, Tonbo Biosciences, San Diego, CA), and anti-CD45 e450 (clone HI30, eBioscience, San Diego, CA) for 30 minutes at 37°C. After incubation, the suspensions were washed with DPBS, fixed with 0.5% neutral buffered formalin (VWR International, West Chester, PA), washed again with DPBS, and run in a BD LSRII flow cytometer. Single antigen beads decorated with different HLAs fluoresce in the PE channel with distinct intensity, allowing one to discern the individual HLA alleles. The abundance of unbound beads was quantified in the presence of either A2-CAR Tregs or untransduced Tregs for each single HLA antigen group. Percentage relative binding of A2-CAR Tregs to each HLA allele was then calculated using the following formula

$$\frac{100 \times (norm.\ number\ beads\ in\ UT\ Treg - norm.\ number\ beads\ in\ CAR\ Treg)}{norm.\ number\ beads\ in\ UT\ Treg}$$

i.e. by dividing the normalized (norm.) number of beads in the untransduced (UT) Treg condition for a specific HLA minus the normalized number of beads in the A2-CAR Treg condition for that same HLA by the normalized number of beads in the untransduced Treg condition, multiplied by 100. HLA antigen bead numbers were normalized using the following formula

$$\frac{200 \times number\ beads}{number\ negative\ control\ beads}$$

i.e. by multiplying the number of beads of interest in each HLA peak by 200, divided by the number of negative control beads in the sample, to correct for variations in the absolute number of negative control beads acquired in each sample.

Genome Engineering

CRISPR/Cas9 genome editing in Tregs and bulk T cells was carried out using ribonucleoprotein (RNP) electroporation as previously described (37). Briefly, RNPs were produced by complexing a two-component guide RNA (gRNA) to Cas9. crRNAs and tracrRNAs were chemically synthesized (Dharmacon, IDT) and Cas9-NLS (nuclear localization signal) was recombinantly produced and purified (QB3 Macrolab). Lyophilized RNA was resuspended at a concentration of 160 μM, and stored in single use aliquots at −80°C. crRNA and tracrRNA aliquots were thawed, mixed 1:1 by volume, and annealed at 37 °C for 30 min. 40 μM recombinant Cas9 was mixed 1:1 by volume with the 80 μM gRNA (2:1 gRNA to Cas9 molar ratio) at 37 °C for 15 min to form an RNP complex at 20 μM. RNPs were electroporated immediately after complexing into Tregs and T cells resuspended in supplemented P3 buffer (Lonza).

Guide RNA sequences used for gene editing were:

TRAC: CAGGGTTCTGGATATCTGT
TRBC: CCCACCAGCTCAGCTCCACG
HLA-A2: CCTCGTCCTGCTACTCTCGG

In **Figure 1**, A2-CAR lentivirus alone was used to engineer Tregs. In **Figures 2**, **3** (and **Supplementary Figures 4–6**), the TCR was removed using a constant region of the TCR beta chain (*TRBC*)-specific CRISPR/Cas9 RNP and an A2-CAR lentivirus to engineer the CAR Tregs. Lentivirus was transduced at a multiplicity of infection (MOI) of 1 by spinoculation for 30 min at 1200 G. The next day, cells were washed to remove

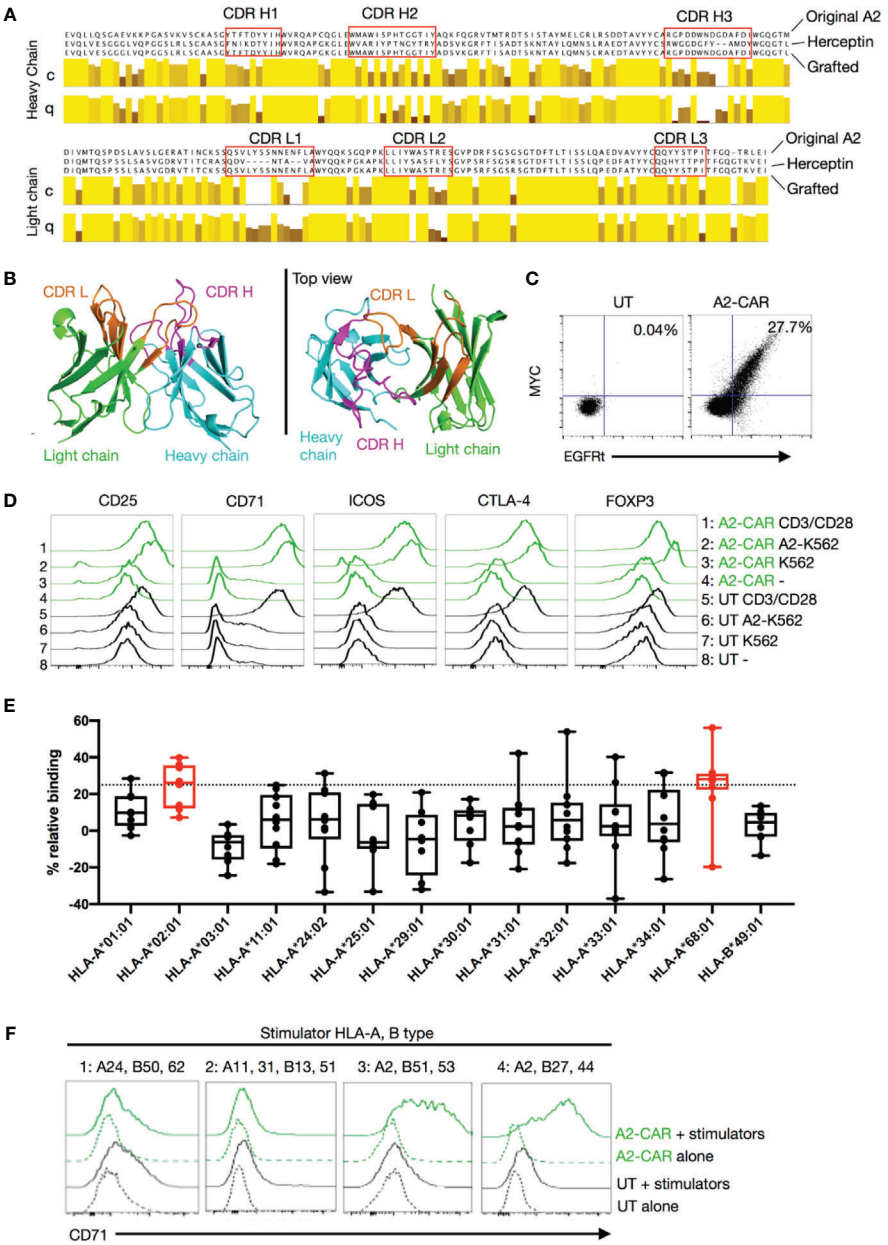

FIGURE 1 | Generation of a grafted A2-CAR. **(A)** Grafting strategy comparing the original V_H and V_L chain sequences of the SN607D8 hybridoma and of the Herceptin (trastuzumab) 4D5 scaffold. The grafted amino acid sequences are shown. The sequences of SN607D8 and 4D5 HER2 were aligned using the software Jalview and the level of conservation (C) and quality (Q) of each amino acid between SN607D8 and 4D5 sequences were compared. Conservation reflects similarity of the physicochemical properties of amino acid residues. Identical residues are shown as light-yellow columns and residues with more dissimilar physicochemical properties are marked with darker column colors. Quality measures the likelihood of observing a mutation in any particular amino acid residue position (38). CDRs were predicted using Paratome (29). **(B)** The conformation of the grafted antibody was predicted with ABodyBuilder (39) and displayed using PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA). **(C)** EGFRt and MYC-tag expression on Day 6 of culture of human Tregs transduced with the grafted A2-CAR-2A-EGFRt lentivirus. **(D)** On Day 9, A2-CAR Tregs were cultured for another 48 hours alone, with anti-CD3/CD28 beads, or with irradiated (4000 rad) parental $A2^-$ K562 or A2-expressing K562 cells. CD25, CD71, ICOS, CTLA-4, and FOXP3 expression were analyzed thereafter using flow cytometry. **(E)** OneLambda FlowPRA Single HLA Antigen bead panels FL1HD01 and FL1HD02. Percentage relative binding of A2-CAR Tregs to each HLA allele was calculated as described in the Materials and Methods section. Plotted averages of at least 5 independent experiments. Red coloring indicates HLA allele beads surpassing the 25% binding threshold to be considered binders. **(F)** On Day 9, A2-CAR or UT T cells were cultured for another 48 hours alone or with dissociated islet cells from 4 allogeneic donors. Expression of CD71 was analyzed thereafter using flow cytometry. HLA-A and -B alleles expressed by the 4 allogeneic donors are indicated above the histograms. For donors 1, 3, and 4, A2-CAR and UT Tregs were used in the assay and for donor 2, A2-CAR and UT $CD4^+$ Tconv cells are used. Results are a summary of 4 independent experiments using T cells from unrelated healthy donors. *scFv, single-chain variable fragment; CDR, complementarity-defining region; A2, HLA-A2; UT, untransduced; Tconv, conventional T cells*.

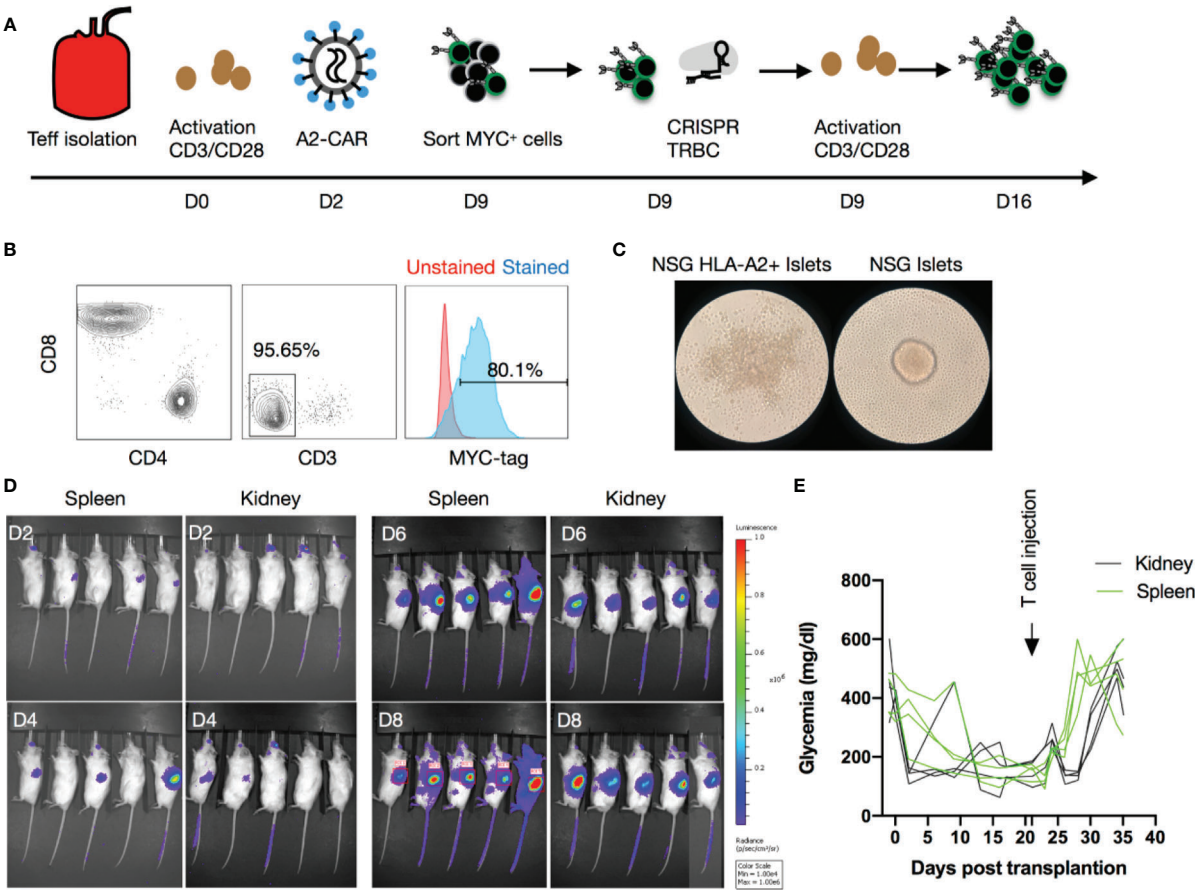

FIGURE 2 | Human A2-CAR T cell trafficking and function in an islet transplantation model. **(A)** Experimental design of human T-cell engineering and expansion. **(B)** Editing efficiency as measured by CD3 and MYC-tag expression on T cells prior to infusion from an HLA-A2 negative donor after CRISPR editing of the TCR (*TRBC*) and transduced with a lentivirus consisting of a A2-CAR linked to a luciferase reporter gene by a 2A self-cleaving peptide. **(C)** WT NSG or HLA-A2 transgenic NSG mouse islets pictured 48h after co-culture with A2-CAR T cells (100 IEQ with 1 x 10^5 T cells). Result shown is representative of two experiments using Tregs from 3 unrelated donors. **(D)** Luciferase activity over time after infusion of 2 x 10^6 A2-CAR T cells in mice that received an A2 transgenic murine islet transplant either under the left kidney capsule or into the spleen. **(E)** Glycemia monitoring after streptozotocin (STZ) injection, islet transplantation, and T-cell infusion (2 x 10^6/mouse from a HLA-A2 negative donor). An insulin pellet was placed subcutaneously in mice with partial graft function (blood glucose >200mg/dl) on Day 10 after transplantation.

residual virus from the medium and further expanded with recombinant human IL-2 (300IU/ml). In **Figure 2**, A2-CAR$^+$ cells were FACS-purified on Day 9 based on MYC-tag expression and the TCR was deleted by electroporating a CRISPR/Cas9 RNP complex targeting *TRBC* thereafter. In **Figure 3**, the TCR was deleted after Treg/Teff cell purification and prior to Treg/Teff cell activation with anti-CD3/CD28 beads.

In **Figures 4–6**, two days after anti-CD3/CD28 bead-mediated activation, cells were electroporated with a *TRAC*-specific and an HLA-A2-specific CRISPR/Cas9 RNP (1:1 ratio, final volume 5 µl). Tregs and T cells were replated for expansion together with an AAV6 containing the A2-CAR homology-directed repair (HDR) template. The next day, the cells were washed to remove residual virus from the medium and further expanded with recombinant human IL-2 (300IU/ml). In this case, the *HLA-A2* gene was removed, as A2-CAR Tregs cannot be engineered from HLA-A2 positive donors (data not shown).

A2-CAR Treg Trafficking to Transplanted Pancreatic Islets

Female or male NSG mice were rendered diabetic by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) at 220mg/kg and islets were transplanted 72-96 hours later. Blood glucose levels were monitored 2-3 times per week using a glucometer (Nova Max Plus Blood glucose and Ketone Monitor, Nova Diabetes care, Billerica, MA). Only mice with blood glucose levels above 300mg/dl were used for transplantation. Pancreatic islets from NSG.HLA-A2 transgenic mice (A2-NSG, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(HLA-A/H2-D/B2M)1Dvs/SzJ, Jackson Laboratories, Bar Harbor, ME, Stock number 014570) were isolated as previously described (40). Human

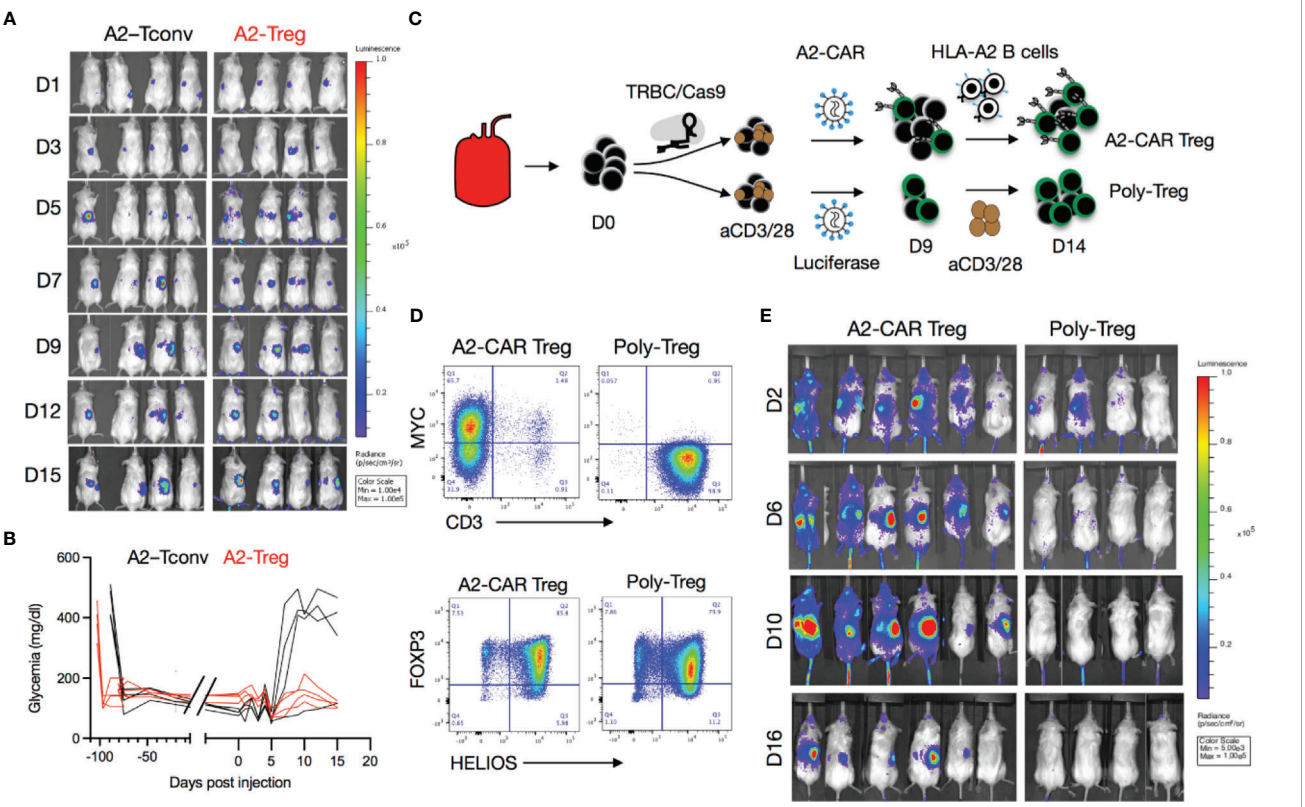

FIGURE 3 | Trafficking of A2-CAR Tregs to islet grafts. **(A)** TCR deficient (*TRBC*-CRISPR edited), lentiviral transduced, 2 x 10^6 A2-CAR CD4$^+$ Tconv cells or A2-CAR Tregs, (harvested on Day 10 after sorting from peripheral blood of an HLA-A2 negative donor) were infused into mice that had been stably transplanted with HLA-A2 transgenic mouse islets. Luciferase activity of A2-CAR CD4$^+$ Tconv cells or A2-CAR Tregs over time is shown. **(B)** Glycemia was monitored over time after cell infusion. **(C)** Tregs were edited with CRISPR/Cas9 ribonucleoprotein (RNP) complexes targeting the *TRBC* locus after cell sorting, activated with anti-CD3/28 beads, and transduced with a lentivirus consisting of an A2-CAR linked to a luciferase reporter gene by a 2A self-cleaving peptide two days later. On Day 9, Tregs were re-stimulated with HLA-A2$^+$ stimulated B cells (sBCs) for another 5 days and were thereafter injected in diabetic mice (2 x 10^6/animal) transplanted with HLA-A2$^+$ human islets. In parallel, polyclonal Tregs were activated with anti-CD3/28 beads, transduced with a lentivirus expressing a luciferase reporter gene alone, and restimulated with anti-CD3/28 beads on Day 9 and injected on Day 14 in diabetic mice (2 x 10^6/animal) transplanted with HLA-A2$^+$ human islets. **(D)** Editing efficiency was measured by MYC-tag and CD3 surface expression in two independent donors. Treg purity was assessed by FOXP3 and HELIOS expression in the same donors. **(E)** Luciferase activity of A2-CAR Tregs and polyclonal Tregs (transduced with a lentivirus expressing a luciferase reporter alone) over time.

pancreata were procured from deceased multi-organ donors with research use consents and approval from UCSF institutional review board. Human research islets were isolated by the UCSF Diabetes Center Islet Core following standard protocols (41). A total of either 500 mouse islets or 3000 human islet equivalents (IEQs) were transplanted under the kidney capsule or into the spleen. Blood glucose levels of < 200mg/dl on two consecutive days were defined as successful islet engraftment. Mice that only attained partial graft function (blood glucose range 200-500mg/dl) by 10 to 14 days after transplant were given subcutaneous insulin pellets (Linbit, LinShin Canada) to support graft function. Luciferase-expressing A2-CAR Tregs or A2-CAR T cells were infused intravenously in STZ-induced diabetic mice transplanted with mouse HLA-A2$^+$ islets. Luciferase activity was monitored 2-3 times per week. These animals were anesthetized in an isofluorane chamber, injected i.p. with 100 µl of 15 mg/ml D-Luciferin (Biosynth, Staad, Switzerland) and, 7 min later, imaged in a Xenogen IVIS Spectrum Imaging System (PerkinElmer, Richmond, California). Luciferase data analysis was performed using Living Image software (PerkinElmer).

Xenogeneic Graft-*vs*-Host Disease

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) and NOD.Cg Prkdc^{scid} Il2rg^{tm1Wjl}/Tg(HLA-DRB1)31Dmz/SzJ/H2-Ab1tm1Gru x NOD.Cg-Tg(HLA-A/H2-D/B2M)1Dvs/SzJ (A2-NSG) were obtained from Jackson Laboratories. For GvHD induction, animals were irradiated (2.5Gy) 24 hours prior to retroorbital intravenous (i.v.) infusion of 5 x 10^6 freshly isolated PBMCs from either an HLA-A2-positive or an HLA-A2-negative donor with or without 2.5 x 10^6 *ex vivo* expanded third-party A2-CAR Tregs. All mouse experiments were performed according to a UCSF Institutional Animal Care and Use Committee (IACUC) approved protocol.

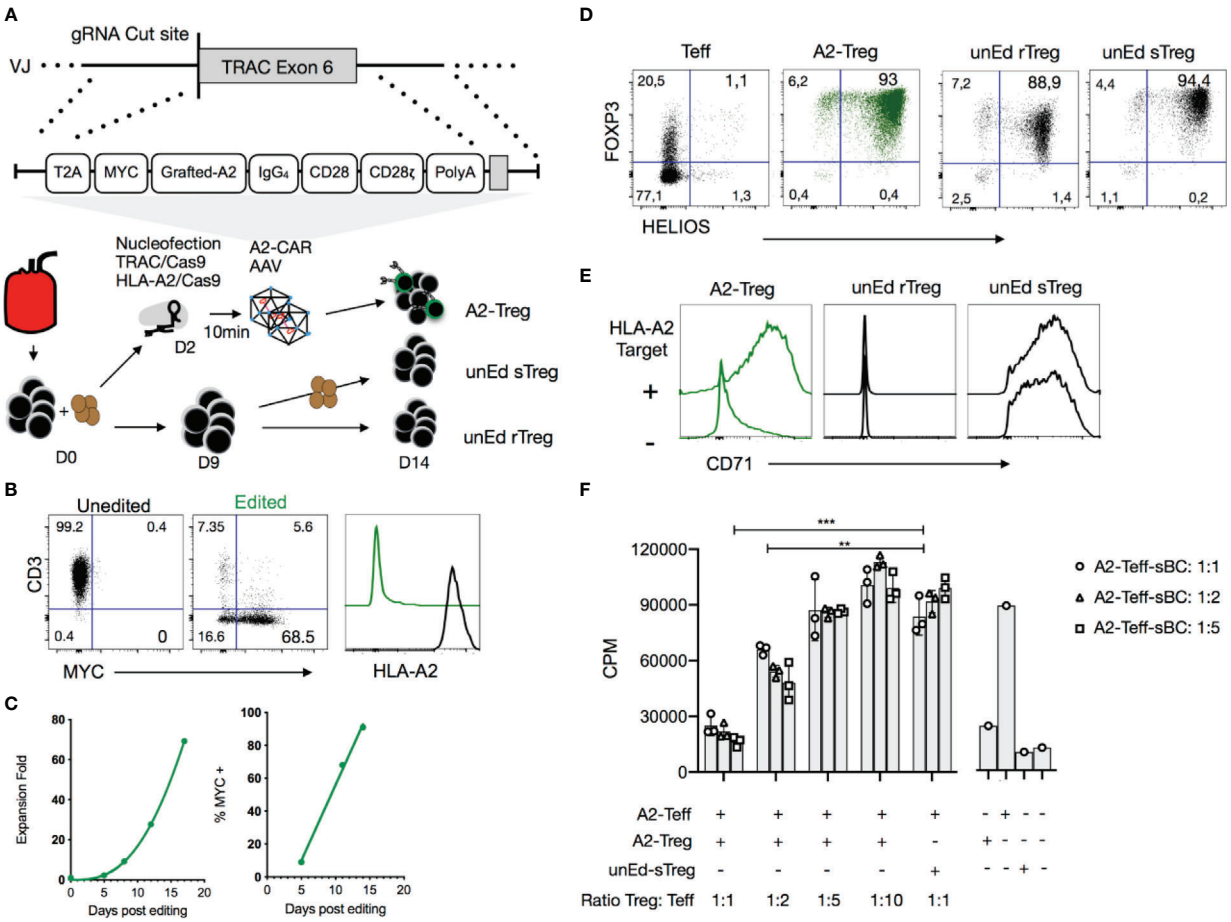

FIGURE 4 | Precision engineering of an A2-CAR into the TRAC locus of human Tregs. **(A)** Expansion strategy of A2-CAR Tregs, unedited resting Tregs (unEd rTreg) and unedited stimulated Tregs after sorting from peripheral blood of an HLA-A2 positive donor. For homology-directed repair-mediated integration into the *TRAC* locus, the A2-CAR template was inserted using AAV6 transduction after electroporation of two CRISPR/Cas9 ribonucleoprotein (RNP) complexes targeting the *TRAC* and *HLA-A* loci. **(B)** Representative flow cytometry of the editing efficiency measured 10 days later in 3 independent experiments. CD3, MYC-tag, and HLA-A2 surface expression is shown. **(C)** Treg fold-expansion and percentage of MYC-tag$^+$ Tregs over time. Fitted line plots are shown. **(D)** Fourteen days after activation, FOXP3 and HELIOS expression were assessed on edited Tregs and compared to that of unedited T cells and unedited Day 9 stimulated Tregs (unEd sTregs). **(E)** The same cells were co-cocultured with or without irradiated (4000 rad) HLA-A2$^+$ NALM6 cells. CD71 expression was assessed 48 hours later. **(F)** *In vitro* suppression assays were performed using HLA-A2$^+$ stimulated B cells (sBCs) as stimulator cells, A2-CAR$^+$TCR^{deficient} CD4$^+$ T cells as responder cells (0.05 x 10^6 cell/96well) and A2-CAR$^+$TCR^{deficient} Tregs at different ratios. After 3 days, 0.5μCi/well of ^{3}[H] thymidine (Perkin Elmer, Waltham, MA) was added for the final 16 h of culture. Proliferation was assessed by ^{3}[H] thymidine incorporation (counts per minute - cpm). Two-way ANOVA was used to determine the statistical significance of the difference. Data corresponds to the cells infused in **Figure 5**. Similar results were obtained with 3 independent donors. ***p < 0.01; ***p < 0.001. Teff, CD4$^+$ T effector cells; A2-Treg, A2-CAR$^+$TCR^{deficient} Treg; unEd sTreg, unedited Day 9 stimulated Tregs; A2-Teff, A2-CAR$^+$TCR^{deficient} Teff; sBC, stimulated B cells; CPM, counts per minute.*

RESULTS

Development of an HLA-A2-Specific CAR

To engineer an anti-A2 CAR, we first cloned the variable regions of the heavy (V_H) and light (V_L) chains of an A2-specific IgG1κ antibody from a hybridoma (SN607D8) produced using B cells isolated from a previously described sensitized donor (24). This antibody was reported to bind to HLA serotypes A2 and A28 (broad antigen which includes HLA-A68 and A69 alleles as split antigen). After cloning the SN607D8 scFv from the hybridoma, we evaluated phage-displayed SN607D8 scFv binding to two human tumor cell lines. The THP-1 monocytic cell line expresses HLA-A2, but not A28, whereas the RPMI 8226 myeloma cell line is HLA-A2$^-$ but has a genotype of HLA-A*6802 and is thus HLA-A28$^+$. The results showed that the SN607D8 scFv indeed binds to both cell lines (**Supplementary Figure 1**), demonstrating the retention of the original specificity of the antibody.

We then cloned the SN607D8 scFv into a construct that contained an IgG4 hinge, the CD28 transmembrane domain, and a signaling domain composed of the CD28 and CD3ζ intracellular domains. Unexpectedly, the CAR failed to express on the surface of human T cells (data not shown). To rescue the expression, we grafted the complementarity-determining regions

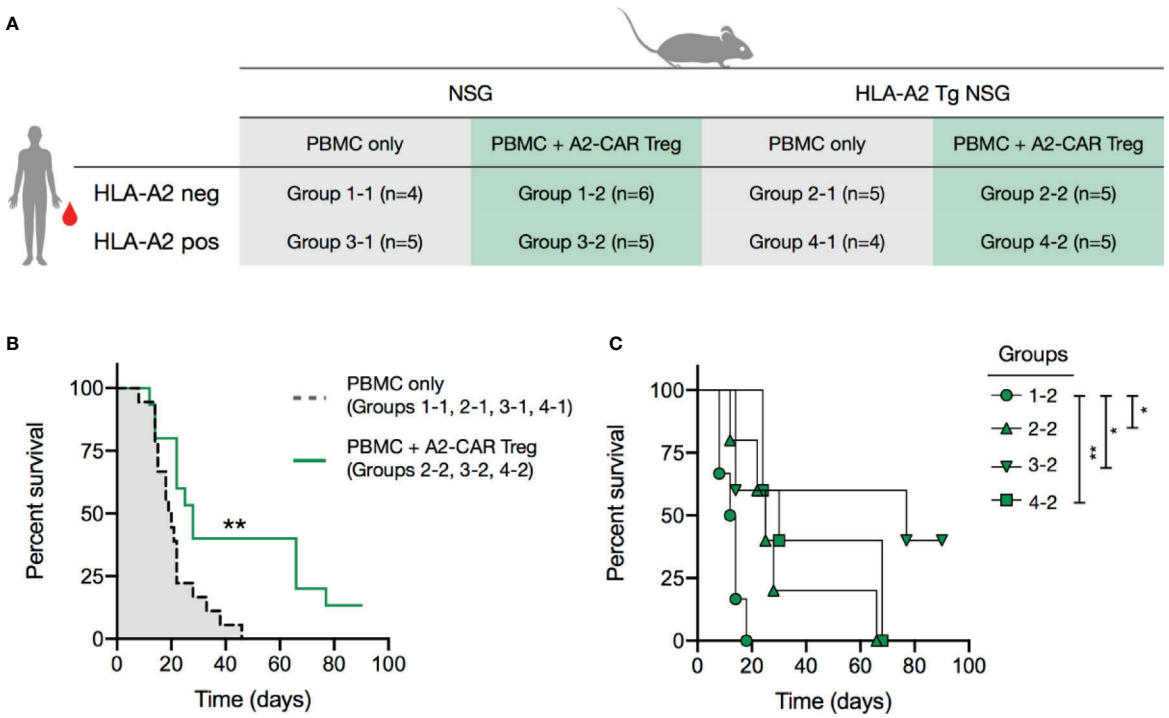

FIGURE 5 | A2-CAR Tregs confer protection against graft-*versus*-host disease. **(A)** 2.5 x 10^6 AAV CRISPR-edited A2-CAR$^+$TCR^{deficient} Tregs (generated as described in **Figure 4**) were co-injected with 5 x 10^6 A2 negative or positive PBMC in wild type or A2 transgenic immunodeficient NSG mice. PBMCs and Tregs were injected into contralateral retro-orbital plexus. **(B)** Overall survival of mice that received PBMCs (A2 positive or negative, NSG A2 positive or negative) or PBMCs and A2-CAR$^+$TCR^{deficient} Tregs, excluding the condition where Tregs remained unstimulated (group 1-2). **(C)** Overall survival of mice treated with A2-CAR$^+$TCR^{deficient} Tregs comparing NSG PMBC (group 1.2, round), A2-NSG PBMC (group 2-2, triangle), NSG A2$^+$ PBMC (group 3-2, inverted triangle, n=6), and A2-NSG A2$^+$ PBMC (group 4-2, square). Log-rank (Mantel-Cox) test was used for statistical analysis *$p < 0.05$, **$p < 0.01$.

(CDRs) of the heavy and light chains of the SN607D8 scFv into the framework regions of an scFv derived from the anti-HER2 antibody Herceptin (trastuzumab), which is known to be compatible with CAR surface expression (42). The resulting grafted heavy and light chains (**Figure 1A**) were connected *via* a 15 amino acid linker $(GGGGS)_3$ to form a new grafted scFv, termed QT007YL. Automated computer modeling with an antibody structure prediction tool, ABodyBuilder (39), showed that the grafted scFv folds as expected (**Figure 1B**). We then generated a new A2-CAR for expression in human T cells by fusing the QT007YL scFv to an IgG4 hinge, the CD28 transmembrane domain, and a CD28-ζ signaling domain. The resulting A2-CAR was cloned with an N-terminal MYC-tag into a pCDH lentiviral vector behind an EF1α promoter (30). A truncated EGFR (EGFRt) was cloned in-frame behind the CAR separated by a 2A self-cleaving peptide to enable facile evaluation of lentiviral transduction using expression of EGFRt. To enable *in vivo* tracking of CAR-expressing cells, we also generated a version of the lentiviral construct with a luciferase gene behind the CAR separated by a 2A peptide (**Supplementary Figure 2A**). We first transduced HLA-A2-negative Jurkat T cells to assess the expression and function of the grafted A2-CAR. Detection of the MYC-tag *via* flow cytometry verified efficient surface expression of the A2-CAR (**Supplementary Figure 2B**). Lentiviral transduction of primary human Tregs with the grafted A2-CAR also resulted in co-detection of the MYC-tag and EGFRt on the cell surface (**Figure 1C**). Moreover, A2-CAR Jurkat T cells upregulated CD69 and CD25 expression specifically when co-cultured with irradiated HLA-A2$^+$ K562 tumor cells (**Supplementary Figure 2C**), suggesting that the grafted A2-CAR is able to activate T-cell signaling in response to HLA-A2. Finally, we evaluated A2-CAR-mediated *in vitro* activation of human Tregs following co-culture with either HLA-A2$^+$ or HLA-A2$^-$ K562 cells (**Figure 1D**). A2-CAR Tregs upregulated CD25, CD71, ICOS, FOXP3, and CTLA4 48 hours after stimulation with HLA-A2$^+$, but not with HLA-A2$^-$ cells.

The HLA-A2 molecule contains 41 polymorphic epitopes (called eplets) that are shared with other HLA class I molecules (**Supplementary Table 2**). The parental monoclonal antibody SN607D8 used to generate the grafted A2-CAR has a specificity for the eplet 144TKH (142T, 144K, and 145H residues), which is shared among HLA-A2, -A68, and -A69, but not with HLA-A3, -A11, or -A24 (43). To verify that the grafted A2-CAR QT007YL retained the specificity for 144TKH, we tested binding of grafted A2-CAR transduced Tregs to a FlowPRA Single Antigen bead panel. In this assay, CAR specificity is defined as an increase in binding to HLA-bearing beads over control beads of at least 25%, a threshold used to define the binding specificity of a previously

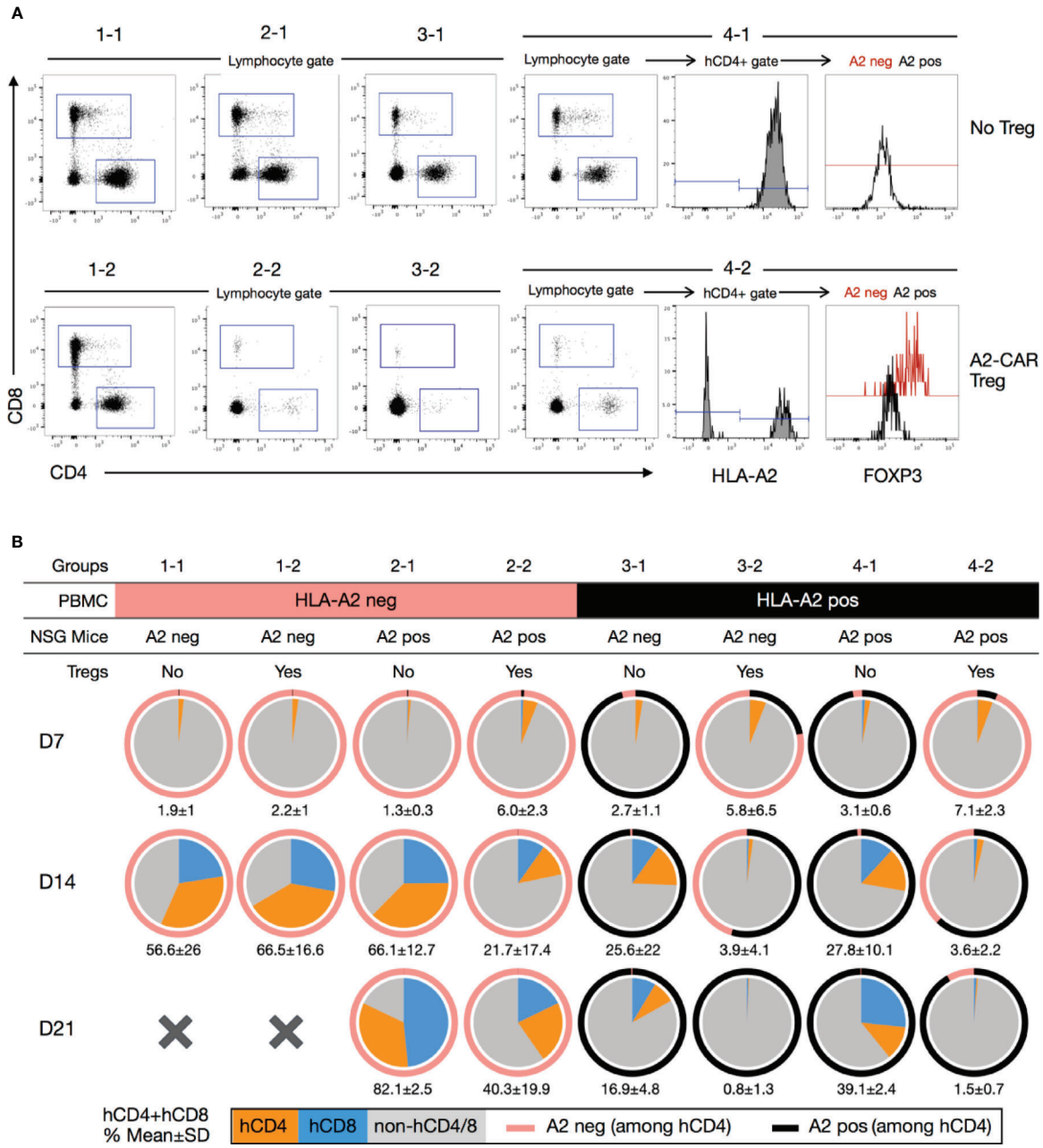

FIGURE 6 | Blood monitoring of immune reconstitution in xenogeneic graft-*versus*-host disease. **(A)** Representative staining of human CD8$^+$, human CD4$^+$ and hCD4$^+$A2^{neg}FOXP3$^+$ subpopulations after dead cell (Ghost fixable viability dye positive) exclusion. Representative example of mice from each group (1-1, 1-2, 2-1, 2-2, 3-1, 3-2, 4-1, 4-2). **(B)** Pie charts showing the mean percentage of human CD4$^+$ (orange) and CD8$^+$ (blue) cells in the peripheral blood of mice 7 (n=4-6), 14 (n=2-5) and 21 days (n=2-4) after injection. The numbers below each pie chart represent the mean ± standard deviation of the combined percentage of human T cells (hCD4$^+$ and hCD8$^+$). *A2, HLA-A2*.

reported A2-CAR (36). The grafted A2-CAR reacted with HLA-A2 and HLA-A68, but not HLA-A3, -A11, -A24, -A25, -A30-A34 (**Figure 1E**). This pattern of reactivity ruled out all other eplets but the two overlapping eplets of 144TKH and 144KHA, consistent with the specificity of the parental antibody. To further demonstrate the specificity of the QT007YL A2-CAR for HLA naturally expressed on human cells, we incubated A2-CAR T cells or Tregs with dissociated HLA-typed primary human islet cells and measured their activation 48 hrs later by CD71 upregulation. A2-CAR$^+$ cells were activated by stimulator cells expressing HLA-A2, but not those expressing HLA-A11, -A24, or -A31 (**Figure 1F**). These results, together with the

FlowPRA data, demonstrate that the QT007YL A2-CAR retained the specificity of the parental clone SN607D8.

In Vivo Trafficking of Monospecific A2-CAR T Cells in an Islet Transplant Model

Next, we injected QT007YL A2-CAR-expressing T cells in NSG mice to determine whether CAR expression could redirect T cells to HLA-A2-expressing tissues *in vivo*. A significant fraction of human T cells can recognize mismatched HLA and trigger rejection of transplanted allogeneic human tissue in NSG mice (44). Additionally, human T cells have conspicuous reactivity against xenogeneic antigens expressed in the mouse host, with the potential to divert T cells away from human grafts and also eventually cause GvHD (45). To avoid these confounding issues, we first lentivirally transduced primary human T cells to express the A2-CAR. We subsequently generated A2-CAR^{+}TCR^{deficient} T cells by CRISPR/Cas9-mediated knockout of endogenous TCR expression from Day 9 FACS-purified A2-CAR^{+} cells (**Figure 2A**). After editing, we restimulated the cells with anti-CD3/CD28 beads for another 7 days, resulting in 95.65% CD3^{-} and 80.1% MYC-tag^{+} cells (**Figure 2B**). Co-culturing these TCR^{deficient} A2-CAR T cells with islets from HLA-A2 transgenic NSG (A2-NSG) or WT NSG mice for 48 hours resulted in the selective destruction of A2-NSG transgenic mouse islets (**Figure 2C**), demonstrating that the grafted A2-CAR can be specifically activated by HLA-A2 molecules expressed on islet tissue *in vitro*.

To determine if TCR^{deficient} A2-CAR T cells can recognize A2-expressing islets *in vivo*, we first transplanted HLA-A2 transgenic mouse islets into STZ-induced diabetic NSG mice, and then infused the mice with 2 x 10^6 TCR^{deficient} A2-CAR T cells after the islet grafts had been established. The kidney capsule is a standard site for islet transplantation. However, human CD4^{+} T cells efficiently trafficked to the lungs, livers and spleens, but not kidneys of NSG recipients (**Supplementary Figure 3**). It has been previously reported that infusion of 2 x 10^7 allogeneic human PBMCs at the time of islet transplant can lead to rejection of human islets transplanted under the kidney capsule in NSG mice within 3 weeks. However, after the graft has been stably engrafted, 2 x 10^7 allogeneic human PBMCs cannot consistently reject the islets before the mice succumb to GvHD due to the xenogeneic response of the human PBMCs against the mouse hosts (46). We reasoned that, at the time of islet transplant, ischemic injury to the islets led to the release of chemokines and other inflammatory mediators to attract the PBMCs. Once islet grafts are established and the inflammation subsides, PBMCs may not efficiently traffic to the kidney to mediate the rejection. Primary human islets from deceased donors become available for research with very short (< 2 days) advanced notice and need to be transplanted right away to ensure optimal function. This, unfortunately, does not allow time to produce CAR T cells, so we had to rely on the use of a pre-engrafted islet model. To improve the chance of A2-CAR T cell encounter with the islets, we transplanted the islets into the spleen of the NSG mice and included the standard left kidney capsule site for comparison. Three weeks after transplantation, we infused the mice with luciferase-expressing A2-CAR T cells and monitored their migration using bioluminescence imaging. We observed progressive increase in luciferase signal in the recipient mice, although it was difficult to discern the accumulation of A2-CAR T cells in the spleen *versus* the left kidney (**Figure 2D**). Moreover, we observed a sharp synchronous rise in blood glucose first among mice with islet grafts in the spleen, followed by mice with islets grafted in the kidney capsule, indicative of rejection of the A2-NSG islets. The median survival was 6 days in the spleen and 11 days under the kidney capsule (**Figure 2E**), indicating that A2-CAR T cells trafficked to the transplanted kidney to mediate the rejection. To further substantiate graft-specific trafficking of A2-CAR T cells, we transplanted A2 transgenic murine islets either in the liver, the spleen, under the left or left kidney capsule. Luminescence signals were detected only in the locations corresponding to sites of the islet grafts (**Supplementary Figure 4**). Altogether, these data demonstrated that the A2-CAR was able to direct the trafficking and accumulation of human T cells to sites of antigen deposition outside the default route of T cell migration.

We then used the kidney capsule islet transplantation model to evaluate the *in vivo* trafficking of A2-CAR Tregs. Luciferase-labeled human TCR^{deficient} A2-CAR Tregs were generated as described in **Figure 2A** and the resulting cells expressed Treg lineage markers FOXP3 and CD25 (**Supplementary Figure 5**). We then infused these cells into STZ-induced diabetic animals transplanted with A2-NSG transgenic islets. In this experiment, the islets were transplanted under the right kidney capsule to enable greater spatial separation from the spleen during bioluminescence imaging. To support Treg persistence in the absence of human IL-2-producing cells, mice infused with 2 x 10^6 A2-CAR Tregs received daily i.p. injections of recombinant human IL-2 (50,000 IU/day/mouse). For comparison, we separately infused a cohort of mice with 2 x 10^6 TCR^{deficient} A2-CAR conventional CD4^{+} T cells (Tconv), without IL-2 injection. In both groups, luciferase activity was observed first in the spleen and 3-7 days later in the right kidney (**Figure 3A**) demonstrating that both A2-CAR Treg and Tconv cells can traffic to the A2^{+} grafts. Importantly, islet rejection was observed in 3 out of 4 mice that received A2-CAR CD4^{+} Tconv cells, but none of the 4 mice that received A2-CAR Tregs (**Figure 3B**). Despite accumulation within the grafts, the lack of mouse islet destruction by the human A2-CAR Tregs suggests that CAR Tregs do not have overt toxicity against islets.

To validate that A2-CAR Tregs can traffic to human A2^{+} islets, we repeated this experiment with human HLA-A2^{+} islets transplanted under the right kidney capsule. In this experiment, human Tregs were first treated with CRISPR/Cas9 ribonucleoprotein (RNP) complexes targeting the TCR beta constant (*TRBC*) locus to eliminate endogenous TCR expression prior to lentiviral transduction with an A2-CAR-2A-luciferase construct. To expand sufficient numbers of TCR^{deficient} A2-CAR Tregs, cells were re-stimulated with HLA-A2^{+} stimulated B cells (sBCs) on Day 9 of culture for an additional 5 days (**Figure 3C**). As a control, TCR-unedited polyclonal Tregs were transduced with a luciferase-only

construct and restimulated with anti-CD3/28 beads on Day 9. Tregs maintained FOXP3 and HELIOS expression prior to infusion (**Figure 3D**). Mice received 2 × 10^6 Tregs intravenously (i.v.) with subsequent daily i.p. IL-2 injections. A2-CAR-expressing, but not polyclonal Tregs, trafficked from the spleen to the right kidney (**Figure 3E**). Together, these results demonstrate that $TCR^{deficient}$A2-CAR Tregs efficiently traffic to and accumulate in human HLA-$A2^+$ islets *in vivo*.

Knocking the A2-CAR Into the TRAC Locus of Tregs

To investigate A2-CAR function independently of the endogenous TCR, we employed homology-directed repair (HDR) to site-specifically integrate the A2-CAR into the TCR alpha constant (*TRAC*) locus, replacing expression of the endogenous TCR with expression of the A2-CAR (**Figure 4A**). $CD4^+CD25^{high}CD127^{low}$ Tregs were FACS-purified and activated with anti-CD3/CD28 beads in the presence of IL-2 (300 IU/ml). Two days later, anti-CD3/CD28 beads were magnetically removed, and the cells were electroporated with Cas9-gRNA ribonucleoprotein (RNP) complexes targeting the *TRAC* locus and transduced with AAV6 encoding the QT007YL A2-CAR HDR template. Because this blood donor happened to be HLA-$A2^+$, we also included Cas9-gRNA RNP designed to target the *HLA-A2* gene locus. *TRAC* and *HLA-A2* gene knockout efficiencies were approximately 85% and 95%, respectively. A minor (5%) population of MYC-tag^+TCR^+ cells was observed, likely resulting from incomplete *TRAC* inactivation and either monoallelic A2-CAR genomic integration into the other *TRAC* locus or off-target integration (**Figure 4B**). Importantly, while the percentage of A2-CAR-expressing MYC-tag^+ (edited) Tregs was initially low (9%), A2-$CAR^+TCR^{deficient}$ Tregs preferentially expanded *in vitro*, presumably due to activation by residual HLA-A2 surface expression shortly after CRISPR/Cas9-mediated *HLA-A2* gene knockout. In the absence of further exogenous stimulation, 91% of cells were MYC-tag^+ after 14 days (**Figure 4C**).

On Day 14 of culture, we evaluated FOXP3 and HELIOS expression among edited A2-$CAR^+TCR^{deficient}$ Tregs, unedited $CD4^+$ Tconv, and unedited Tregs [unEd sTreg, stimulated by anti-CD3/CD28 beads on Days 0 and 9, as per our standard protocol for polyclonal Treg expansion (47)]. Over 99% of A2-$CAR^+TCR^{deficient}$ Tregs were FOXP3 positive and 93% were HELIOS and FOXP3 double positive (**Figure 4D**). Co-culture of the A2-$CAR^+TCR^{deficient}$ Tregs (A2-CAR Treg) with NALM6, an HLA-A2-positive B cell-derived leukemia cell line, led to a marked and specific upregulation of surface CD71 expression, demonstrating the antigen-driven activation of the edited cells. Unedited resting Tregs (unEd rTreg) remained CD71 negative, whereas anti-CD3/28 bead-stimulated unedited Tregs (unEd sTreg) were CD71 positive regardless of the presence of NALM6, as expected (**Figure 4E**). Finally, we evaluated the suppressive function of the A2-$CAR^+TCR^{deficient}$ Tregs *in vitro* by co-culturing them with A2-$CAR^+TCR^{deficient}$ T cells and HLA-$A2^+$ irradiated sBCs and assessing T cell proliferation. A2-$CAR^+TCR^{deficient}$ Tregs suppressed the proliferation of A2-$CAR^+TCR^{deficient}$ T cells in the presence of HLA-$A2^+$ sBCs, whereas unedited polyclonal Tregs did not (**Figure 4F**). The lack of suppression observed with polyclonal Treg may be explained by the low frequency of allogenic Tregs which could interact with A2-expressing B cells and by the strong activation mediated by the CAR in responder T cells, as previously reported (48, 49).

Monospecific A2-CAR Treg Function *In Vivo* in Xenogeneic GvHD Models

We next tested the *in vivo* functionality of A2-$CAR^+TCR^{deficient}$ Tregs *in vivo* within models of xenogeneic GvHD, induced by human PBMCs in sub-lethally irradiated NSG mice. In these experiments, we induced GvHD by infusing PBMCs from an HLA-$A2^+$ or an HLA-$A2^-$ donor into HLA-A2-transgenic or wild-type (i.e. lacking HLA-A2 expression) NSG mice (**Figure 5A** and **Supplementary Figure 6A**). This created 4 experimental groups with regards to the expression of HLA-A2: 1 absent; 2 expressed by the NSG recipients, 3 expressed by the infused PBMCs; and 4 expressed by both NSG recipients and PBMCs. A subset of mice in each of the 4 groups also received A2-$CAR^+TCR^{deficient}$ Tregs at the time of PBMC infusion. We first confirmed in a mixed lymphocyte reaction (MLR) that A2-$CAR^+TCR^{deficient}$ Tregs upregulated CD71 only in the presence of the PBMCs from the HLA-$A2^+$ donor used for the immune reconstitution (**Supplementary Figure 6B**). To avoid direct contact between the PMBCs and Tregs during infusion, we injected the cells separately into contralateral retro-orbital plexus. A2-$CAR^+TCR^{deficient}$ Tregs delayed GvHD in mice that had HLA-A2 expressed by the PBMCs, the NSG recipients, or both (**Figure 5B**) and failed to confer any protection against GvHD in wild-type NSG animals reconstituted with HLA-$A2^-$ PBMC, with a median survival of 13 days (**Figure 5C**). This demonstrates that A2-CAR Tregs can function in the absence of the endogenous TCR and that A2-CAR Treg-mediated protection from GvHD depends on the presence of the HLA-A2 antigen.

To further investigate the mechanism of A2-$CAR^+TCR^{deficient}$ Treg-mediated protection, we determined the percentage of circulating human $CD4^+$ and $CD8^+$ T cells in the peripheral blood of treated animals at Days 7, 14, and 21 following cell injection (**Figure 6A**). As previously reported (18), we observed that HLA-$A2^+$ T cells barely engrafted in mice that also received A2-$CAR^+TCR^{deficient}$ Tregs, irrespective of HLA-A2 expression by the host mice (**Figures 6A, B**). Meanwhile, the frequency of engrafted HLA-$A2^-$ T cells was vastly reduced, but not completely eliminated, when co-injected with A2-$CAR^+TCR^{deficient}$ Tregs in HLA-$A2^+$ transgenic host mice. In HLA-A2 transgenic mice reconstituted with HLA-$A2^+$ PBMCs (groups 4-1 and 4-2), we found that the circulating HLA-$A2^-$ cells, i.e. the engineered A2-$CAR^+TCR^{deficient}$ Tregs, remained $FOXP3^+$ (**Figure 6A**), albeit with limitations in the number of acquired events due to the marked decrease in the number of $CD4^+$ and $CD4^+$HLA-$A2^-$ cells over time (**Figure 6B**).

DISCUSSION

Here, we report the successful development of a novel human anti-HLA-A2 CAR. Two other human and humanized A2-CARs have been previously described: one by the group of Megan Levings, where a mouse anti–A2 BB7.2 hybridoma was humanized (36), and a second by the groups of Giovanna Lombardi and Elmar Jaeckel, generated from a previously published anti-HLA-A2 antibody sequence (50) (clone 3PB2 V_H and DPK1 V_L) derived from a sensitized blood transfusion patient (19, 20). Our original anti-HLA-A2 hybridoma (SN607D8), first described in 2003, was isolated from a woman sensitized during her pregnancies (24). Its HLA specificity was determined by complement-dependent cytotoxicity on a large panel (n>230) of HLA-typed peripheral blood lymphocytes (51), where it was found to cross-react with HLA-A28, a split antigen that encompasses HLA-A68 and HLA-A69 alleles. The epitope responsible for this cross-reactivity has also been pinpointed, being defined by the amino acids 142T/145H (52). Such extensive characterization was instrumental for us to confirm the preservation of the specificity of the original and the grafted A2-CAR scFv.

Our initial failure to express an A2-CAR constructed with an scFv derived from the original SN607D8 hybridoma on the surface of human T cells suggests possible conformational instability. We then grafted the scFv CDR regions into an scFv framework (trastuzumab) known to be compatible with CAR surface expression (42). Thus, the trastuzumab framework may confer greater stability to scFvs for CAR protein folding and expression. However, it should be noted that we have not tested this grafting strategy with other scFvs, and thus cannot be certain of the broader applicability of this CDR-grafting approach. Nevertheless, our success in grafting the specificity of the SN607D8 antibody shows that this approach may be useful when designing CARs for new targets.

It is currently unknown whether the endogenous TCR impacts the function of CAR Tregs. Thymically derived Tregs have a highly diverse TCR repertoire that is skewed towards recognizing autoantigens (53), and work in mice has demonstrated that Tregs require continuous TCR signaling to maintain normal immune homeostasis (54). Thus, retaining the TCR in CAR Tregs might support their homeostasis *in vivo*. However, in the context of solid organ transplantation, A2-CAR Tregs traffic to the HLA-A2-expressing graft, as shown in previous work (19, 20) and in this study, thus receiving continuous signaling *via* the CAR, ultimately inducing bystander suppression and supporting homeostasis independently from their endogenous TCR. Our islet transplantation experiments show that A2-CAR Tregs with or without endogenous TCR efficiently traffic to the site of antigen expression. Moreover, the finding that TCR-deficient A2-CAR$^+$ Tregs suppress GvHD, in an HLA-A2-dependent manner, shows that CAR Tregs can function without their endogenous TCR.

Our results are consistent with previous work that has shown efficient GvHD prevention by A2-CAR Tregs in NSG mice infused with HLA-A2$^+$ PBMCs (18). By analyzing PBMC engraftment, it was apparent that the protection from GvHD was a result of preventing T cell engraftment, possibly due to direct recognition of HLA-A2$^+$ PBMCs by A2-CAR Tregs. Yet, low T cell engraftment is not a desirable outcome of Treg therapy for GvHD. On the contrary, immune reconstitution, and subsequent recovery of protective immunity, is needed to safeguard cancer patients who receive a bone marrow transplant against infectious agents and residual cancer cells (55, 56). Our results show that A2-CAR Tregs can delay GvHD not only when the PBMCs themselves express HLA-A2, but also when HLA-A2 is expressed by the recipients and not by the PBMCs.

In the conditions of HLA-A2$^+$ PBMC and A2-CAR Treg co-transfer (group 3-2 and 4-2), we were able to track the infused A2-CAR Tregs by their hCD4$^+$HLA-A2$^-$ phenotype. We observed that A2-CAR Tregs dominated in the first week, but their percentages among human CD4$^+$ T cells (pink portion of the outer rings of the pie charts) were reduced by the 2nd week and barely detectable by the 3rd week. The cause of poor A2-CAR Treg persistence is not clear, but likely secondary to low human T cell engraftment, thus limited IL-2 availability, needed for Treg survival. The lack of A2-CAR Treg persistence might allow the residual human T cells unopposed by Tregs to recover, eventually resulting in lethal GvHD in these mice.

One potential benefit of eliminating endogenous TCR expression is to more precisely control Treg specificity, especially in the context of universal CAR Tregs for off-the-shelf use. Creating and banking such universal CAR Tregs may circumvent the challenge of expanding Tregs from immunosuppressed transplant recipients and the long production time that precludes their use in acute conditions. In this vein, we show in this study that we can simultaneously ablate HLA-A2 and TCR expression at high efficiency in human Tregs, while maintaining stable FOXP3 and HELIOS expression, as well as antigen-specific suppressive function *in vitro* and *in vivo*. Recently, progress has been made towards the generation of universal human pluripotent stem cells, which portend potential inexhaustible sources of universally compatible cells, tissues, and organs for therapy (57, 58). Our data support the feasibility of developing universal engineered Tregs with precisely controlled specificity while evading host immune destruction to induce immune tolerance. Future experiments aimed at further characterizing the suppressive potency and longevity of CAR Tregs *in vivo* will shed additional light on the efficiency, safety, and feasibility of this strategy for Treg-based cell therapy.

In conclusion, we demonstrated that it is feasible to engineer a grafted CAR directly into the *TRAC* locus of human Tregs. This strategy is highly efficient, does not cause Treg destabilization, and allowed for the generation of Tregs with CAR-restricted specificity that delayed GvHD in a target antigen-dependent manner. This strategy can be applied for precision engineering of therapeutic Tregs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC, University of California, San Francisco.

AUTHOR CONTRIBUTIONS

Designed the project: QT and JB. Supervised the project: QT, JB, and JW. Designed experiments: YM, LF, KL, YZ, and QT. Performed experiments: YM, LF, ER, PH, GF, VN, KL, YZ, NS, and AK. Analyzed data: YM, LF, and QT. Provided reagents and advice: AM and FC. Wrote the manuscript: YM, LF, and QT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Highly Purified Alloantigen-Specific Tregs From Healthy and Chronic Kidney Disease Patients Can Be Long-Term Expanded, Maintaining a Suppressive Phenotype and Function in the Presence of Inflammatory Cytokines

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The adoptive transfer of alloantigen-specific regulatory T cells (^{allo}Tregs) has been proposed as a therapeutic alternative in kidney transplant recipients to the use of lifelong immunosuppressive drugs that cause serious side effects. However, the clinical application of ^{allo}Tregs has been limited due to their low frequency in peripheral blood and the scarce development of efficient protocols to ensure their purity, expansion, and stability. Here, we describe a new experimental protocol that allows the long-term expansion of highly purified allospecific natural Tregs (nTregs) from both healthy controls and chronic kidney disease (CKD) patients, which maintain their phenotype and suppressive function under inflammatory conditions. Firstly, we co-cultured CellTrace Violet (CTV)-labeled Tregs from CKD patients or healthy individuals with allogeneic monocyte-derived dendritic cells in the presence of interleukin 2 (IL-2) and retinoic acid. Then, proliferating $CD4^+CD25^{hi}CTV^-$ Tregs (allospecific) were sorted by fluorescence-activated cell sorting (FACS) and polyclonally expanded with anti-CD3/CD28-coated beads in the presence of transforming growth factor beta (TGF-β), IL-2, and rapamycin. After 4 weeks, ^{allo}Tregs were expanded up to 2,300 times the initial numbers with a purity of >95% ($CD4^+CD25^{hi}FOXP3^+$). The resulting allospecific Tregs showed high expressions of CTLA-4, LAG-3, and CD39, indicative of a highly suppressive phenotype. Accordingly, expanded ^{allo}Tregs efficiently suppressed T-cell proliferation in an antigen-specific manner, even in the presence of inflammatory cytokines (IFN-γ, IL-4, IL-6, or TNF-α). Unexpectedly, the long-term expansion resulted in an increased methylation of the specific demethylated region of *Foxp3*. Interestingly, ^{allo}Tregs from both normal

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individuals and CKD patients maintained their immunosuppressive phenotype and function after being expanded for two additional weeks under an inflammatory microenvironment. Finally, phenotypic and functional evaluation of cryopreserved ^{allo}Tregs demonstrated the feasibility of long-term storage and supports the potential use of this cellular product for personalized Treg therapy in transplanted patients.

Keywords: transplantation, regulatory T cells, allospecific, expansion, suppression

INTRODUCTION

Kidney transplantation is currently the therapy of choice for most causes of chronic kidney disease (CKD) (1). To prevent allograft rejection, kidney transplant (KT) patients are treated with immunosuppressive drugs that reduce the rates of renal acute rejection and increase patients' survival (2). However, the long-term use of immunosuppressants can have adverse side effects in KT patients, such as the increase of neoplasias, infections, and cardiovascular diseases, which in turn represent some of the main causes of death in these patients (3). Therefore, it is still necessary to develop new therapies that induce specific allograft tolerance.

Regulatory T cells (Tregs) have a crucial role in establishing and maintaining peripheral immune tolerance. Tregs are characterized by the expression of FOXP3, a transcription factor that regulates their immunosuppressive function (4). Studies in mouse models have demonstrated that Tregs are essential to inducing specific KT tolerance by the inhibition of effector T cells and modulating dendritic cell function (5). KT patients who developed clinical operational tolerance exhibit a significant increase in the number of circulating FOXP3$^+$ Tregs (6, 7). Hence, the clinical application of Tregs has been extensively studied as an approach for the induction of allotransplant tolerance (8).

Due to the low frequency of Tregs in peripheral blood (<1% of white blood cells), several protocols for *ex vivo* expansion of Tregs have been designed to obtain the cell numbers required for immunotherapy (8). Following this approach, clinical trials using polyclonal Tregs have been implemented in humans, with hematopoietic stem cell transplantation (HSCT) reporting a significant decrease of the severity of graft *versus* host disease (GvHD) (9, 10). Similarly, phase I or phase I/IIa studies adopting expanded Tregs have recently been performed in KT patients (11–13), which reported neither infusion-related side effects nor increased infections or rejection events during post-transplant, evidencing the safety of Treg therapy. Nonetheless, these studies have used polyclonal Tregs, and preclinical studies have indicated that adoptive transfer of alloantigen-specific Tregs (^{allo}Tregs) may be a better approach to inducing long-term allograft acceptance (14–17).

The high precursor frequency of natural Tregs (nTregs) recognizing alloantigens directly (5%–10% of blood Tregs) compared with indirectly (<0.1%) (18) has promoted the development of Treg expansion protocols based on direct allorecognition (19). However, the large-scale production of human allospecific Tregs for immunotherapy has remained a challenge due to the lack of optimized protocols to allow their purification and efficient expansion, preserving their functional and phenotypic stability (19). In addition, studies have shown that long-term expansion of Tregs results in the loss of FOXP3 and may convert to potentially inflammatory T cells (20, 21). Recently, our group has applied a protocol that allows the generation of a large number of functionally stable allogeneic induced Tregs (iTregs) after long-term polyclonal expansion (22). Finally, another important issue recently addressed to optimize the function of infused Tregs for the induction of effective tolerance toward the allograft is the homing capabilities of the infused Tregs (23).

In the present study, we describe a new protocol where the increased expansion and survival of long-term stimulated ^{allo}Tregs allows the production of highly purified allospecific Tregs from healthy individuals and patients with CKD that maintain a suppressive phenotype and suppressor function in the presence of pro-inflammatory cytokines, supporting the potential of *in vitro* expanded allospecific Tregs for immunotherapy in kidney transplantation.

MATERIALS AND METHODS

Patients With Chronic Kidney Disease

The present study was approved by the Committees of Medical Ethics and Research at the Instituto de Investigaciones Biomédicas (UNAM) and the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (reference #1831) and was performed in accordance with the revised Declaration of Helsinki, the Declaration of Istanbul, and Good Clinical Practice Guidelines. All patients provided written informed consent to participate in the study and were maintained in renal replacement therapy while awaiting kidney transplant.

Buffy coat preparations of blood from healthy individuals (control group) were provided by the Blood Bank of the Instituto Nacional de Enfermedades Respiratorias, México.

Reagents and Antibodies

For flow cytometry, allophycocyanin (APC) anti-CD4, PerCP-Cy5.5 anti-CD4, PE-Cy7 anti-CD8, APC anti-CD11c, phycoerythrin (PE) anti-CD86, fluorescein isothiocyanate (FITC) anti-CD14, and Foxp3/Transcription Factor Staining Buffer Kit were obtained from Tonbo Biosciences (San Diego, CA, USA). Alexa Fluor 647 anti-FOXP3 was from Beckman Coulter (Brea, CA, USA). PE-Cy5.5 anti-CD3 was from

Invitrogen (Waltham, MA, USA). APC-Cy7 anti-human leukocyte antigen DR isotype (HLA-DR), Brilliant Violet 711 anti-CD39, Brilliant Violet 421 anti-CTLA-4, PE anti-CD25, PE-Cy7 anti-CD127, PE-Cy7 anti-LAG-3, FITC anti-Helios, and Zombie Aqua™ were purchased from Biolegend (San Diego, CA, USA).

For *in vitro* experiments, rapamycin, retinoic acid, Ficoll® Paque Plus, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (San Louis, MO USA). Recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interleukin 2 (IL-2), IL-4, IL-6, transforming growth factor beta (TGF-β), and tumor necrosis factor alpha (TNF-α) cytokines were from PeproTech (Rocky Hill, NJ, USA). Carboxy fluorescein succinimidyl ester (CFSE), CellTrace Violet (CTV), Dynabeads Human T-Activator CD3/CD28 (anti-CD3/anti-CD28-coated beads), DynaMag-5™ Magnet (DynaMag), CTS™ OpTmizer™ T Cell Expansion SFM medium (expansion medium), RPMI 1640 medium, antibiotic–antimycotic 100×, L-glutamine (GlutaMAX™), sodium pyruvate (100 mM), Minimum Essential Medium non-essential amino acids (MEM-NEAA, 100×), and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Pooled human AB serum was obtained from Gemini Bio Products (Sacramento, CA, USA). All culture media were supplemented with L-glutamine, sodium pyruvate, MEM-NEAA, and antibiotic-antimycotic. The cultures of T cells were performed in round bottom 96-well culture plates (Corning, Avon, France).

Isolation and Cryopreservation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy individuals, patients with CKD, and their potential living kidney donors by density gradient centrifugation over Ficoll® according to the manufacturer's instructions. A portion of PBMCs was resuspended in a cold freezing medium (10% DMSO and 90% FBS) at a concentration of 10^6 cells/ml, stored for 24 h at −70°C, and then transferred to liquid nitrogen. For functional assays, the cells were thawed in a 37°C water bath, washed twice with RPMI medium supplemented with 10% FBS, and resuspended in culture medium.

Monocyte-Derived Dendritic Cells

$CD14^+$ monocytes were purified from PBMCs using the Human CD14 MicroBeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Isolated $CD14^+$ monocytes from kidney donors or healthy controls were cultured in RPMI medium supplemented with 10% human AB serum and stimulated with IL-4 (50 ng/ml) and GM-CSF (50 ng/ml) for 8 days. On days 3 and 5, the culture medium and cytokines (25 ng/ml of IL-4 and GM-CSF) were refreshed. On day 8, monocyte-derived dendritic cells (Mo-DCs) were washed twice with the culture medium and irradiated 3000 rad before the functional assays. A proportion of Mo-DCs was stained with anti-CD14, anti-CD86, anti-CD11c, anti-HLA-DR, and Zombie Aqua™. Then, the cells were acquired on the Attune NxT Flow Cytometer (Thermo Fisher Scientific) and the data analyzed with FlowJo vX.0.7 software (Tree Star, Covington, KE, USA).

Isolation and Expansion of Allospecific Tregs

For the isolation of Tregs, PBMCs were stained with anti-CD4, anti-CD127, and anti-CD25 monoclonal antibodies for 20 min at 4°C in the dark, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. $CD4^+CD25^{hi}CD127^-$ and $CD4^+CD25^-CD45RA^+$ gates (**Figure S1A**) were used for sorting Tregs and naive T cells, respectively, using a BD FACSAria I cell sorter. Isolated $CD4^+CD25^{hi}CD127^-$ Tregs were labeled with CTV (5 μM) according to the manufacturer's instructions. Then, CTV-labeled Tregs (2.5×10^4 cells/well) were co-cultured with irradiated allogeneic Mo-DCs (DC/Treg ratio of 1:2) for 7 days in expansion medium with 10% human AB serum, IL-2 (500 U/ml), and retinoic acid (10 nM). On day 7 of co-culture, the cells were stained with anti-CD25, anti-CD4, and Zombie Aqua™ for 20 min at room temperature in the dark. Live proliferating $CD4^+CD25^+CTV^-$ Tregs (allospecific Tregs) (**Figure S1B**) were sorted using a MoFlo XDP cell sorter, collected in RPMI medium with 20% FBS, and cultured for 3 days in expansion medium supplemented with IL-2 (50 U/ml) plus 10% human AB serum. Then, the allospecific Tregs were polyclonally expanded using a modified protocol described previously (21, 22). Briefly, the allospecific Tregs (2.5×10^4 cells/well) were stimulated with anti-CD3/anti-CD28 beads (bead/Treg ratio of 1:2) for 4 days in expansion medium with 10% human AB serum, IL-2 (300 U/ml), TGF-β (2.5 ng/ml), and rapamycin (100 nM). Then, the beads were removed with DynaMag and the cells rested for 3 days in expansion medium with IL-2 (50 U/ml) plus 10% human AB serum. Three additional rounds of stimulation/resting (7 days each) were performed (**Figure 1**). A proportion of allospecific Tregs expanded for 4 weeks were cryopreserved Section *Isolation and Cryopreservation of PBMCs*. For stability assays, on day 28 of polyclonal expansion, the Tregs were stimulated for two additional rounds of stimulation/resting with anti-CD3/anti-CD28 beads (bead/Treg ratio of 1:2) and IL-2 (100 U/ml) in the presence or absence of 10 ng/ml of IFN-γ, IL-4, IL-6, or TNF-α.

In parallel experiments, naive $CD4^+CD25^-CD45RA^+$ T cells were polyclonally expanded using rounds of stimulation/resting with anti-CD3/anti-CD28 beads (bead/T cell ratio of 1:2) and IL-2 (300 U/ml).

Surface and Intracellular Staining of T Cells

Expanded T cells were stained with anti-CD4, anti-CD25, anti-LAG3, anti-CD39, and Zombie Aqua™ for 20 min at room temperature in the dark and washed once with FACS buffer. For intracellular staining, Foxp3/Transcription Factor Staining Buffer Kit was used following the manufacturer's instructions. Briefly, the cells were permeabilized with a fixation/permeabilization solution at room temperature for 1 h, washed with permeabilization buffer 1×, and incubated with anti-FOXP3, anti-CTLA-4, and anti-Helios for 30 min at 4°C in the dark. Samples were acquired on the Attune NxT Flow Cytometer

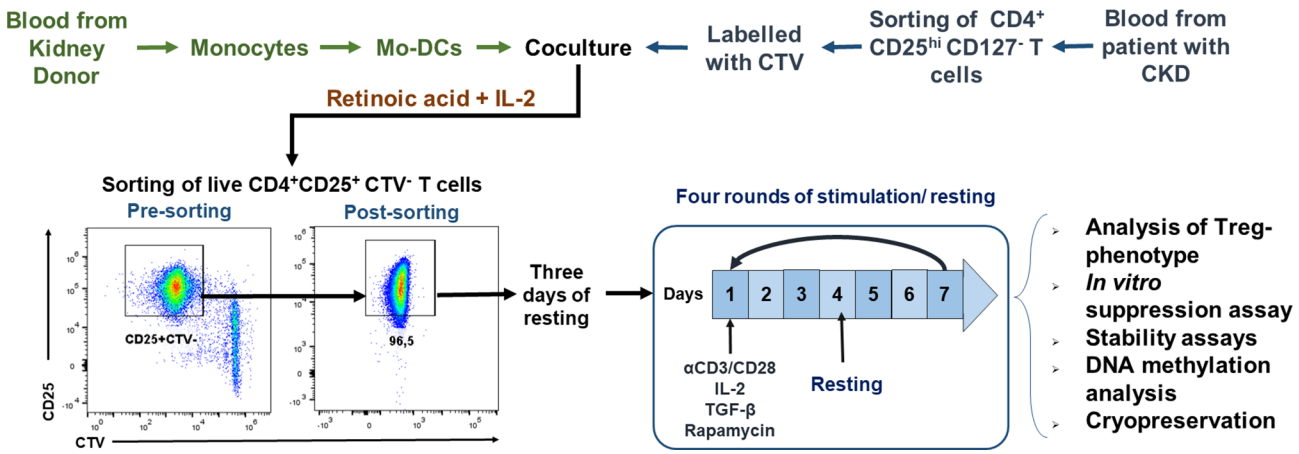

FIGURE 1 | Schematic representation of the protocol used for *ex vivo* expansion of alloantigen-specific regulatory T cells (Tregs). $CD4^+CD25^{hi}CD127^-$ T cells [labeled with CellTrace Violet (CTV)] sorted using fluorescence-activated cell sorting (FACS) were stimulated with allogeneic monocyte-derived dendritic cells (Mo-DCs) in the presence of IL-2 and retinoic acid. On day 7, $CD4^+CD25^{hi}CTV^-$ T cells (allospecific Tregs) were purified by FACS, rested for 3 days, and then polyclonally expanded for 4 days stimulated with anti-CD3/anti-CD28, IL-2, TGF-β, and rapamycin, followed by 3 days resting with only IL-2. After four rounds of polyclonal expansion, analysis of the phenotype and *in vitro* functional assays were performed.

and the data analyzed with FlowJo vX.0.7 software. The median fluorescence intensity (MFI) represented in the graphs was calculated by subtracting the FMO (fluorescence minus one) MFI absolute value of each sample from the respective MFI absolute value. The strategy for the analysis of Tregs by flow cytometry is presented in **Figure S1C**.

In Vitro Suppression Assays

Conventional $CD3^+$ T cells (Tconv) were isolated from PBMCs using the Pan T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions, obtaining purity of $CD3^+$ T cells of >85% (**Figure S2A**). For allospecific suppression assays, expanded Tregs (labeled with CTV) were co-cultured with autologous conventional $CD3^+$ T cells (labeled with 5 μM CFSE, 4×10^4 cells/well) at a Tconv/Treg ratio of 4:1 and the cells stimulated with irradiated allogeneic Mo-DCs (DC/T cell ratio of 1:4) in expansion medium with 10% human AB serum. For stability assays, co-cultures were stimulated in the presence or absence of 10 ng/ml of IFN-γ, IL-4, IL-6, or TNF-α. For polyclonal suppression assays, the co-cultures (at several Treg/Tconv ratios 0:1, 1:2, 1:4, 1:8, and 1:16) were stimulated with anti-CD3/anti-CD28-coated beads (bead/T cell ratio of 1:10) in RPMI medium with 10% human AB serum. After 4 days of co-culture, the cells were stained with anti-CD3, anti-CD4, and anti-CD8 for 20 min at 4°C in the dark, washed twice, and acquired on the Attune Cytometer. The data were analyzed using FlowJo vX.0.7 software. The division index (DI) was determined with CFSE dilution on gated $CD4^+$ or $CD8^+$ T cells, and CTV-labeled Tregs were excluded from the analysis. The strategy for the analysis of the suppression assays is present in **Figure S2B**. The percentage of suppression was calculated using the following formula:

$$\%\ \text{Suppression} = \frac{\text{DI without Treg} - \text{DI with Treg}}{\text{DI without Treg}} \times 100$$

DNA Methylation Analysis of the Treg-Specific Demethylated Region

DNA extraction and sodium bisulfite treatment were performed using the EZ DNA Methylation Direct Kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol. The following primers were used for the PCR amplification of bisulfite-converted genomic DNA: p-5′-TGATTTGTTTGGGGG TAGAGGATTTAGAG-3′ and o-5′-TATCACCCCACCTAAAC CAAACCTACTACA-3′. PCRs were performed on thermocyclers (Thermo Fisher Scientific) in a final volume of 25 μl containing 2.5 μl PCR buffer 10×, 1 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 200 μM dNTPs, 0.4 μM each of forward and reverse primers, and bisulfite-treated genomic DNA. The amplification conditions were 95°C for 15 min and 35 cycles of 95°C for 1 min, 62.5°C for 1 min, and 72°C for 1 min, and a final extension step of 10 min at 72°C. The PCR products were purified using QIAEX II gel extraction kit (Qiagen) and were cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). DH5α competent cells were transformed with recombinant vectors and individual positive bacterial colonies were selected from which recombinant plasmid DNA was purified using FavorPrep Plasmid Extraction Mini Kit (Favorgen, Pingtung, Taiwan). The plasmid DNA was sequenced with 3500 Genetic Analyzer (Thermo Fisher Scientific) and the sequences analyzed using MEGA software v.10.0.5 (Penn State University, State College, PA, USA).

Cytokine Production Assay

For cytokine production analysis, expanded $CD4^+$ T cells (1×10^4 cells/well) were stimulated with anti-CD3/anti-CD28 beads (bead/T cell ratio of 1:1) for 18 h. The levels of cytokines in the culture supernatants were measured using the kit LEGENDplex™ Human Inflammation Panel 1 13-plex

(Biolegend) according to the manufacturer's guidelines. The samples were acquired on the flow cytometer CytoFLEX (Beckman Coulter) and the data analyzed with FlowJo vX.0.7 software. Cytokine concentrations were determined using the standard curve generated in the same assay.

Statistics

Statistical analysis was performed using GraphPad Prism v7.00 software (San Diego, CA, USA). The Shapiro–Wilk test was used to evaluate the distribution of the data. Paired and unpaired *t*-tests were used for comparing normally distributed data; Wilcoxon's rank-sum test or the Mann–Whitney test was used for non-normally distributed data. Differences between more than two groups were calculated using one-way ANOVA or the Kruskal–Wallis test for normally or non-normally distributed data, respectively. Graphs are expressed as mean ± standard error of the mean (SEM). Values with $p < 0.05$ were considered statistically significant.

RESULTS

Allospecific Tregs from CKD Patients Can Be Long-Term Expanded Showing a Highly Immunosuppressive Phenotype

CKD is a pathology characterized by progressive loss of renal function, which will eventually require renal replacement therapy, including kidney transplantation as the best alternative (1). Therefore, patients with CKD could be considered as candidates for Treg-based immunotherapy for the induction of transplant tolerance, alternatively or complementary to the use of immunosuppressive drugs. In the present study, we first evaluated the phenotype of Tregs in peripheral blood from CKD patients and healthy controls, showing similar frequencies of $CD4^+CD25^{hi}CD127^-$ (**Figure S3B**) and $CD4^+CD25^{hi}CD127^-FOXP3^+$ T cells (**Figure S3C**) and no difference in FOXP3 expression (MFI values) within the $CD4^+CD25^{hi}CD127^-$ T-cell population (**Figure S3D**).

With the aim of increasing the yield, purity, and viability of the cellular products obtained with current Treg-based methodologies, we designed a new experimental protocol that allows efficient long-term expansion of highly purified ^{allo}Tregs from both healthy controls and CKD patients (**Figure 1**). Analysis of typical dendritic cell (DC) markers showed that the generated Mo-DCs have high expressions of CD11c, CD86, and HLA-DR and that they do not express CD14 (**Figures S4A–D**). In addition, Mo-DCs induced the *in vitro* proliferation of alloreactive $CD4^+$ and $CD8^+$ T cells from healthy controls at differences ratios of allo-DCs/T cells (**Figure S4E**). Then, purified $CD4^+CD25^{hi}CD127^-$ Tregs were FACS sorted to a purity >95%, labeled with CTV, and co-cultured for a week with allogeneic Mo-DCs. For the initial expansion of allospecific Tregs, we used the combination of retinoic acid (RA) plus IL-2 in the co-cultures, which led to 44.8 ± 9.1% of viable proliferating cells, being $CD4^+CD25^+CTV^-$ Tregs (^{allo}Tregs) (**Figure S5A**). Parallel cultures using IL-2 plus RA alone did not induce significant proliferation of freshly purified $CD4^+CD25^{hi}CD127^-$ Tregs (**Figure S5B**).

Next, FACS-sorted allospecific Tregs from CKD patients and controls were polyclonally expanded for 4 weeks, reaching an increase in the total cell numbers from 1,800- to 2,300-fold (**Figure 2A**). Interestingly, the long-term expanded ^{allo}Tregs from both groups presented a greater proliferation when they were restimulated with the DCs initially used for their expansion (donor DCs) compared to third-party DCs (**Figure S5C**), demonstrating the allospecificity of the expanded ^{allo}Tregs.

Analysis of the Treg phenotype showed that the proportion of $CD25^+FOXP3^+$ cells was significantly increased in expanded ^{allo}Tregs compared to freshly isolated $CD4^+CD25^{hi}CD127^-$ T cells (**Figure 2B**), both in CKD patients (98.7 ± 1.1% *vs.* 89.4 ± 2.5%, $p < 0.01$) and healthy controls (96.3 ± 3.2% *vs.* 88.2 ± 2.0%, $p < 0.05$). Similarly, FOXP3 expression (**Figure 2C**) was significantly upregulated in expanded ^{allo}Tregs from CKD patients (35,586 ± 20,391 *vs.* 7,986 ± 2,384, $p < 0.05$) and controls (27,390 ± 13,692 *vs.* 7,986 ± 2,384, $p < 0.05$). Likewise, the CD25 levels (**Figure 2D**) were significantly increased in ^{allo}Tregs from patients (41,618 ± 27,737 *vs.* 3,399 ± 1,088, $p < 0.05$) and healthy individuals (57,421 ± 24,687 *vs.* 4,101 ± 595, $p < 0.05$). Moreover, no significant differences were found in the proportions of $CD25^+FOXP3^+$ cells and in FOXP3 and CD25 expressions in expanded ^{allo}Tregs from patients compared to Tregs from controls (**Figures 2B–D**).

To further analyze the expanded Treg phenotype, we evaluated the expressions of the molecules related to the suppressive function of Tregs, such as CTLA-4, LAG-3, CD39, and Helios. CTLA-4 expression in Tregs is essential to inhibit the function of antigen-presenting cells (APCs) and the proliferation of conventional T cells *in vitro* and *in vivo* (24). Expanded ^{allo}Tregs from CKD patients showed a high proportion of CTLA-4^+ cells, similarly to ^{allo}Tregs from controls (**Figure 3A**). Furthermore, no significant differences were found in the expression levels of CTLA-4 in the long-term expanded ^{allo}Tregs from patients compared to the ^{allo}Tregs from healthy individuals (**Figure S6A**). LAG-3 is an immunoglobulin superfamily member that has a high affinity to MHC class II molecules expressed on APCs, and this interaction inhibits both the maturation and the antigen-presenting capacity of DCs (25). The expression levels of LAG-3 observed in expanded ^{allo}Tregs from patients were as high as those of ^{allo}Tregs from healthy controls (**Figure 3B** and **Figure S6B**). CD39 is an ectonucleotidase involved in the generation of pericellular adenosine, which inhibits the function of conventional T cells and DCs through the activation of the adenosine 2A receptor (26). Our data showed a high proportion of $CD39^+$ cells in the long-term expanded ^{allo}Tregs from CKD patients and healthy controls (**Figure 3C**). Moreover, no significant differences were found in the CD39 expression of the expanded ^{allo}Tregs from patients compared to the Tregs from controls (**Figure S6C**). Helios is a member of the Ikaros transcription factor family that is important for Treg function (27). The expanded ^{allo}Tregs from both patients and controls displayed heterogeneous proportions of Helios$^+$ cells (**Figure 3D**) and levels of this transcription factor (**Figure S6D**).

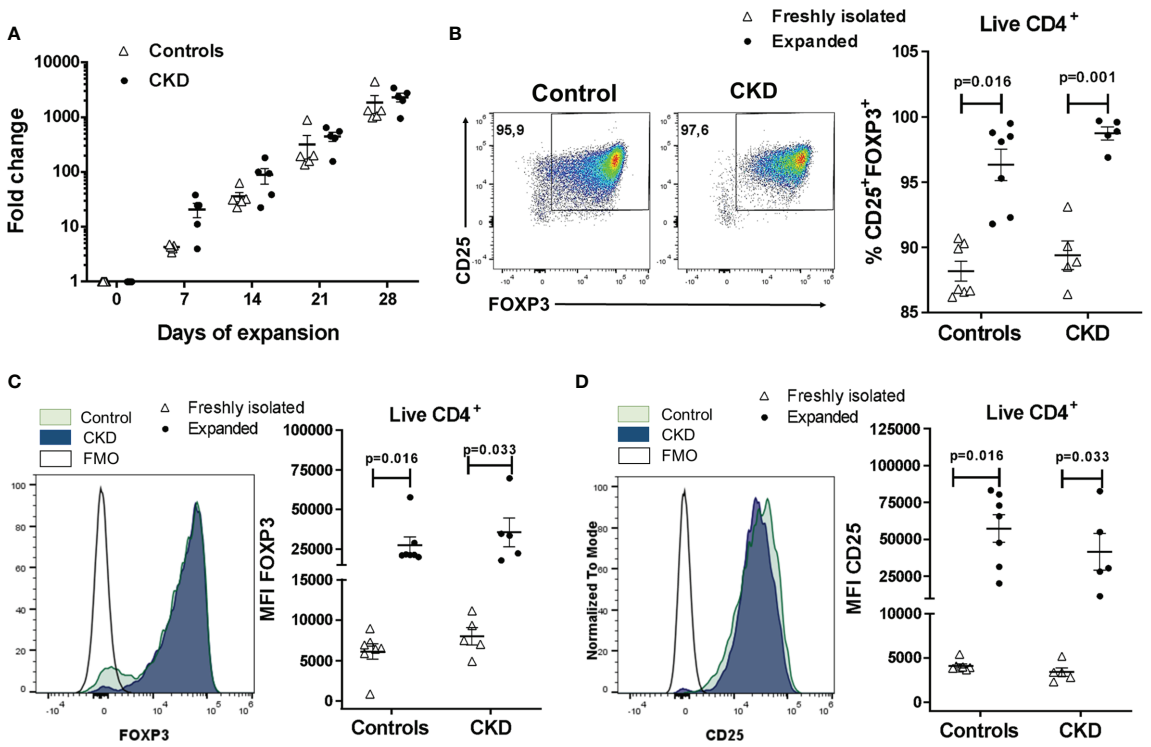

FIGURE 2 | Highly purified allospecific regulatory T cells (Tregs) increased the expressions of FOXP3 and CD25 after *ex vivo* expansion. $CD4^+CD25^{hi}CD127^-$ T cells [labeled with CellTrace Violet (CTV)] from healthy individuals (*white triangles*, $n = 7$) or patients with chronic kidney disease (CKD) (*black circles*, $n = 5$) were stimulated with allogeneic monocyte-derived dendritic cells (Mo-DCs). On day 7, $CD4^+CD25^{hi}CTV^-$ T cells (allospecific Tregs) were purified and polyclonally expanded for 4 weeks; the expressions of FOXP3 and CD25 were evaluated by flow cytometry. **(A)** Allospecific Tregs from patients proliferated to the same extent as the Tregs from healthy controls. Fold expansion was calculated by dividing the number of Tregs obtained on the evaluated day by the number of Tregs on day 0. **(B–D)** The proportion of $CD25^+FOXP3^+$ cells **(B)** and the expressions of FOXP3 **(C)** and CD25 **(D)** were increased in the expanded alloantigen-specific Tregs (^{allo}Tregs) in both study groups compared to those of freshly isolated Tregs. The median fluorescence intensity (MFI) was calculated as described in *Section 2.6*. Representative experiments are shown in **(B–D)**, and the *white histograms* represent FMO (fluorescence minus one) controls **(C, D)**. The results are shown as the mean ± SEM. Statistical analysis was performed using the Mann–Whitney *U* test or Wilcoxon's rank-sum test.

Interestingly, the expanded ^{allo}Tregs from healthy donors showed increased levels of FOXP3 (**Figure S7A**) and higher proportions of $CD25^+FOXP3^+$ (S7B), CTLA-4^+ (S7C), $Helios^+$ (S7D), and LAG-3^+ (S7E) cells compared to expanded naive $CD4^+$ T cells (Tn). To obtain a more detailed profile of the expanded Tregs, we also evaluated cytokine production; importantly, ^{allo}Tregs do not produce significant levels of inflammatory cytokines compared to expanded Tn (**Figure S8**).

Expanded ^{allo}Tregs Efficiently Suppress the Proliferation of Conventional T Cells in an Alloantigen-Specific Manner

After successful expansion of ^{allo}Tregs, we evaluated their suppressive capacity *in vitro* (see *Materials and Methods*). As shown in **Figure 4A**, the expanded ^{allo}Tregs suppressed the proliferation of alloreactive $CD3^+$ Tconv, only when they were stimulated with the DCs toward which they were initially expanded (donor), but not in the presence of DCs from a third party. Moreover, the ^{allo}Tregs from both groups significantly inhibited the proliferation of both $CD4^+$ and $CD8^+$ T cells (**Figure 4B**). Of note is that the allospecific Tregs from CKD patients suppressed the proliferation of alloreactive Tconv to the same extent as that of the Tregs from controls (**Figure 4B**).

Under inflammatory conditions, such as autoimmune diseases and allergy, it has been shown that several cytokines (IL-4, TNF-α, and IL-6) may be involved in downmodulation of Treg suppression (28–30). Thus, it is crucial to assess the functional stability of Tregs for therapeutic purposes. Therefore, we next evaluated the suppressive function of the expanded ^{allo}Tregs *in vitro* in the presence of inflammatory cytokines. As shown in **Figure 4C**, the expanded ^{allo}Tregs from CKD patients and healthy individuals maintained their ability to inhibit the alloantigen-specific proliferation of $CD4^+$ (left) and $CD8^+$ (right) T cells under all inflammatory conditions used. Analysis of the proliferation of Tconv without Tregs in the presence of cytokines showed no significant differences compared to Tconv with media alone (data not shown).

Long-Term Expansion Results in Reduced Demethylation of TSDR-Foxp3 in ^{allo}Tregs

The stability of FOXP3 expression has been shown to correlate with the increased demethylation of the Treg-specific

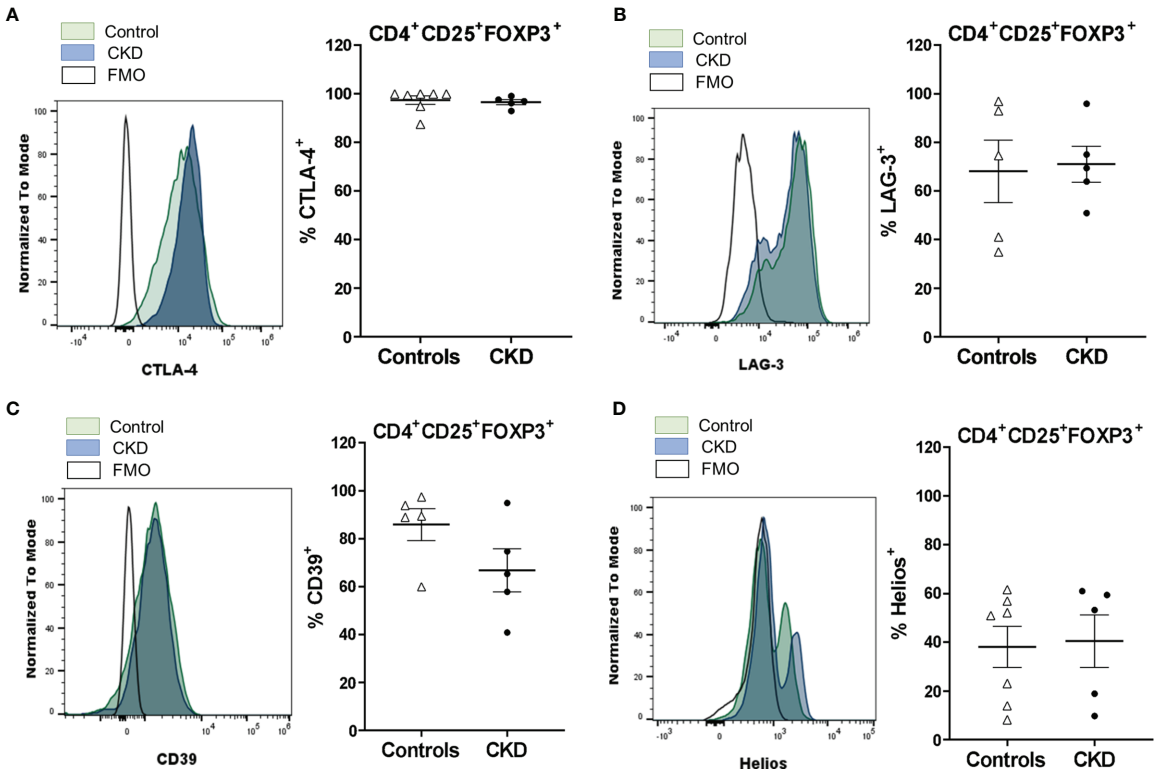

FIGURE 3 | Long-term expanded alloantigen-specific regulatory T cells (^{allo}Tregs) had high expressions of CTLA-4, LAG-3, and CD39. Highly purified ^{allo}Tregs from healthy controls (*white triangles*, *n* = 5-7) or patients with chronic kidney disease (CKD) (*black circles*, *n* = 5) were polyclonally expanded for 4 weeks and then were stained with fluorochrome-conjugated antibodies for analysis by flow cytometry. **(A–D)** Analysis of typical Treg markers showed that the expanded ^{allo}Tregs from both healthy individuals and patients with CKD had high proportions of CTLA-4$^+$ **(A)**, LAG-3$^+$ **(B)**, CD39$^+$ **(C)**, and Helios$^+$ **(D)** cells. Representative experiments are shown **(A–D)**, and the *white histograms* represent FMO (fluorescence minus one) controls. The results are shown as the mean ± SEM. Statistical analysis was performed using the Mann–Whitney *U* test. No significant differences were observed.

demethylated region (TSDR) of the *Foxp3* locus (31). In an attempt to investigate whether the observed phenotype in Tregs was associated with their epigenetic status, we evaluated the methylation of CpG sites in the *Foxp3* gene. Unexpectedly, even though the expanded ^{allo}Tregs showed high FOXP3 expression and suppressive function, the percentage of demethylation of TSDR-*Foxp3* was lower in the expanded Tregs from both groups compared with that in freshly isolated Tregs (**Figure 5**).

^{allo}Tregs Maintain a Suppressive Phenotype After Expansion Under an Inflammatory Microenvironment

One major concern regarding the use of Tregs for immunotherapy is the risk of their conversion into inflammatory T cells and the loss of the suppressive activity during inflammatory responses (32). To gain insight into the stability of Tregs, we evaluated both the phenotype and the *in vitro* function after two additional weeks of polyclonal stimulation with only IL-2 in the presence or absence of inflammatory cytokines. Importantly, the proportion of CD25$^+$FOXP3$^+$ cells (**Figures 6A, B**) and the expressions of FOXP3 (**Figure 6C**) and CD25 (**Figure S9A**) in the expanded ^{allo}Tregs from both patients with CKD and healthy controls were maintained in the presence of all inflammatory cytokines evaluated (IFN-γ, IL-4, IL-6, and TNF-α). Moreover, the stimulation in the presence of inflammatory cytokines did not affect the proportions (**Figures 6D, E**) or the expression levels (**Figures S9B, C**) of CTLA-4$^+$ (**Figure 6D**) and Helios$^+$ (**Figure 6E**) in the expanded ^{allo}Tregs from both groups. Notably, the expanded ^{allo}Tregs from controls and CKD patients maintained >80% FOXP3$^+$CD25$^+$ (**Figure S9D**) and did not significantly reduce their FOXP3 expression levels in the absence of TGF-β and rapamycin (**Figure S9E**).

Subsequently, we evaluated whether the suppressive phenotype of the stimulated Tregs in an inflammatory microenvironment correlated with their *in vitro* function. Indeed, the allospecific Tregs from CKD patients and controls similarly inhibited the proliferation of both CD4$^+$ and CD8$^+$ alloreactive T cells after they were polyclonally expanded for 2 weeks in the presence of inflammatory cytokines (**Figure 7**).

Allospecific Tregs Maintain Their Immunosuppressive Phenotype After Long-Term Cryopreservation

Finally, we investigated the effect of cryopreservation on the phenotype and function of expanded allospecific Tregs. For this

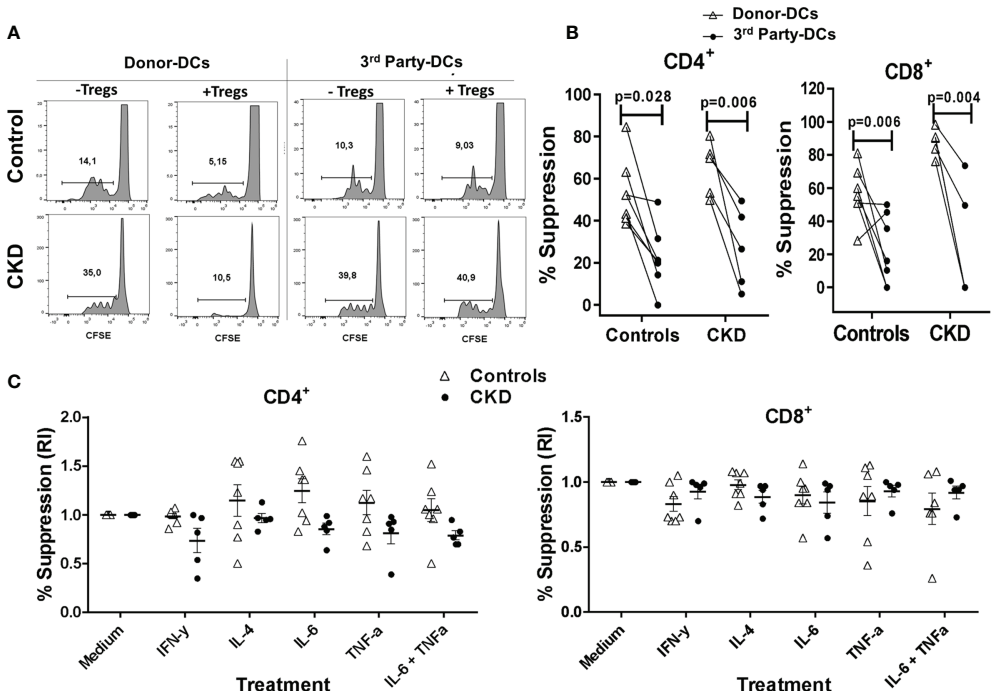

FIGURE 4 | Expanded allospecific regulatory T cells (Tregs) suppressed the proliferation of conventional T cells in an alloantigen-specific manner. Expanded allospecific Tregs (^{allo}Tregs) from healthy individuals (*white triangles*, $n = 7$) or patients with chronic kidney disease (CKD) (*black circles*, $n = 5$) were co-cultured with conventional $CD3^+$ T cells [labeled with carboxy fluorescein succinimidyl ester (CFSE)] and stimulated with allogeneic monocyte-derived dendritic cells (Mo-DCs) from their respective donors or from non-related individuals (third party). On day 4 of culture, T-cell proliferation was evaluated by flow cytometry. **(A)** Representative experiment. **(B)** Expanded ^{allo}Tregs suppressed the proliferation of both $CD8^+$ and $CD4^+$ conventional T cells (Tconv) only when they were stimulated with the dendritic cells (DCs) with which they were initially expanded (DCs from donor), but not when they were stimulated with unrelated DCs (third party). **(C)** ^{allo}Tregs efficiently suppressed alloreactive T cells in the presence of the inflammatory cytokines IFN-γ, IL-4, IL-6, and TNF-α **(C)**. The relative increase (IR) of the percentage of suppression was calculated by dividing the value in the presence of the cytokine by the value in the absence of cytokine (medium). All experiments were performed in duplicate. The results are shown as the mean ± SEM. Statistical analysis was performed using unpaired *t*-test **(B)** or one-way ANOVA **(B)**.

aim, at the end of the expansion, the allospecific Tregs were cryopreserved for a long time (>6 months), and then the cells were thawed and polyclonally expanded. Cryopreserved ^{allo}Tregs from both patients and controls were successfully expanded for 2 weeks, showing higher percentage of $CD25^+FOXP3^+$ cells (**Figure 8A**) and levels of FOXP3 (**Figure 8B**). In addition, the ^{allo}Tregs from both groups have similar percentages of CTLA-4^+ (**Figure 8C**), LAG-3^+ (**Figure 8D**), and $CD39^+$ (**Figure 8E**) cells. Interestingly, when we compared the freshly thawed and expanded Tregs, we found that some suppressive markers (FOXP3, LAG-3, CTLA-4, and CD39) were enhanced after expansion (**Figures 8B–E**). However, the proportion of Helios$^+$ cells was slightly lower in cryopreserved expanded Tregs (**Figure 8F**) compared to that in recently thawed Tregs. In this context, we and others have previously reported the decrease of Helios after *in vitro* stimulation (21, 33).

After successful expansion of cryopreserved Tregs, we investigated the *in vitro* function of these cells. Allospecific Tregs from patients with CKD showed high ability to suppress the proliferation of polyclonal $CD4^+$ and $CD8^+$ T cells, similarly to Tregs from healthy controls, at all evaluated ratios (**Figure 8G**). Moreover, analysis of the expression of chemokine receptor demonstrated that expanded ^{allo}Tregs from both CKD patients and controls showed high expressions of CXCR3, CCR4, and CCR2, recently reported to play an important role in Treg graft homing (23), while ^{allo}Tregs displayed very low levels of CCR7 in both groups (**Figure 9**).

DISCUSSION

In this work, we addressed key issues previously considered to ensure the efficacy and safety of allospecific Treg therapy, including Treg expansion, purity, and stability. To increase the yield of ^{allo}Tregs, FACS-sorted $CD4^+CD25^{hi}CD127^-$ Tregs were expanded in the presence of allogeneic DCs plus RA and IL-2, which increased the numbers of purified ^{allo}Tregs compared to previous reports using rapamycin in primary co-cultures (**Figure S5A**) (18). A previous study had shown that the addition of RA favors the maintenance of the expression of FOXP3 in short-term expanded Tregs (34). In this context, RA significantly promoted the accumulation of H3K9Ac and H3K4me3 in the promoter region and partially decreased the methylation of CpG in the central nervous system (CNS) regions of the *Foxp3* gene locus (35).

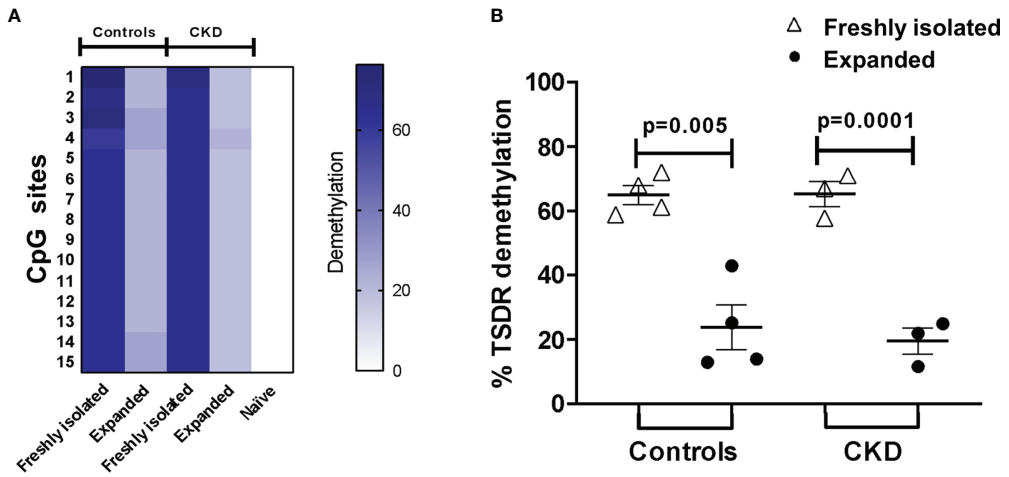

FIGURE 5 | Long-term expansion reduced the demethylation of TSDR-*Foxp3* in purified allospecific regulatory T cells (Tregs). CpG methylation analysis of the Treg-specific demethylated region (TSDR) in the *Foxp3* gene was evaluated in purified $CD4^+CD25^{hi}CD127^-$ Tregs (freshly isolated) and in expanded alloantigen-specific Tregs (^{allo}Tregs). **(A)** Each *square* represents one CpG site analyzed (of a total of 15 CpGs). Six to seven clones per DNA sample were sequenced. The demethylation color code ranges from *white* (0% demethylation) to *navy blue* (80% demethylation), according to the color scale. Purified $CD4^+CD25^-CD45RA^+$ T cells (naive) displayed a completely methylated TSDR and were used as the negative control. **(B)** Freshly isolated Tregs from both groups displayed high demethylation of TSDR-*Foxp3*, while expanded ^{allo}Tregs showed a significant increase in the methylation of the TSDR. DNA samples from three male chronic kidney disease (CKD) patients and four male healthy controls were analyzed. The results are expressed as the mean ± SEM. Statistical analysis was performed using paired *t*-test.

Moreover, RA prevented FOXP3 degradation by downregulating E3 ligase Stub1 expression in activated natural Tregs (35).

To ensure the high purity of ^{allo}Tregs, proliferating Tregs ($CD4^+CD25^+CTV^-$) were isolated by FACS, obtaining a purity of >95% (data not shown). Although most of the current protocols used in Treg immunotherapy are based on immunomagnetic separation, in compliance with good manufacturing practices (GMP) [reviewed in (8)], this process has the limitation of not allowing the use of multiple parameters, including cell proliferation dyes. Additionally, the FACS-based isolation of Tregs has been implemented in clinical trials (36, 37), demonstrating the safety of the cellular product obtained with this approach.

Extensive *in vitro* Treg expansion could result in the loss of FOXP3 expression (20). Previous reports have shown that rapamycin allows the preferential growth of Tregs and maintenance of FOXP3 expression (38, 39), while TGF-β has been linked to the upregulation of FOXP3 through epigenetic modification in the *Foxp3* locus (40, 41). To favor the maintenance of FOXP3 expression during the polyclonal expansion of ^{allo}Tregs, we added both rapamycin and TGF-β to our *in vitro* cultures, obtaining >95% of $CD25^+FOXP3^+$ Tregs after 4 weeks of expansion. With this protocol, we achieved an expansion from 1,800- to 2,300-fold ^{allo}Tregs after 4 weeks of culture (**Figure 2A**), while previous studies reported an expansion ranging from 8- to 780-fold for 12–42 days of culture [revised in (19)]. These results are similar to those obtained in the expansion of allospecific iTregs using a slightly modified protocol, favoring the enrichment of $FOXP3^+$ iTregs (from 60% to >90% of $FOXP3^+$ cells) (22). Moreover, expanded ^{allo}Tregs displayed an increase in the expressions of CD25 and CTLA-4, in correlation with the increase of FOXP3, which directly upregulated the transcription of both molecules by binding to the *Il2ra* and *Ctla4* loci (42). This is functionally relevant as, in the context of transplantation, it has been established that CTLA-4 signaling participates in the early induction of allograft acceptance (24, 43). Importantly, >95% of our expanded ^{allo}Tregs expressed high levels of CTLA-4, while in a previous report (18) only 15% of the ^{allo}Tregs obtained were $CTLA\text{-}4^+$, although the stimulation and staining conditions used in these two studies could account for the differences observed.

Moreover, the ^{allo}Tregs contained high proportions of positive cells for LAG-3 and CD39, which are characteristic suppressive markers of this subpopulation. In the setting of allotransplant, the upregulation of LAG-3 and CD39 on Tregs contributed to prolonging the survival of allografts by modulating the production of inflammatory cytokines in Tconv and the function of DCs (44–47). On the other hand, the ^{allo}Tregs from both patients and healthy controls displayed a heterogeneous expression of Helios, a transcription factor that is required in Tregs to induce the expression of Treg-related genes (48), enhance suppressive function (49), and maintain a stable phenotype during inflammatory responses (50). Although some ^{allo}Tregs showed low expressions of Helios, we (21) and others (33) have described that expanded Tregs may retain their suppressive function despite a reduced Helios expression, suggesting that this marker may not necessarily define the functional status of Tregs.

As some Treg markers can be transiently expressed by activated T cells (51–53), we also analyzed parallel cultures using activated naive $CD4^+$ T cells. Most importantly, ^{allo}Tregs showed highly increased levels of FOXP3 (eightfold), CTLA-4 (sevenfold), and LAG-3 (threefold) compared to those in

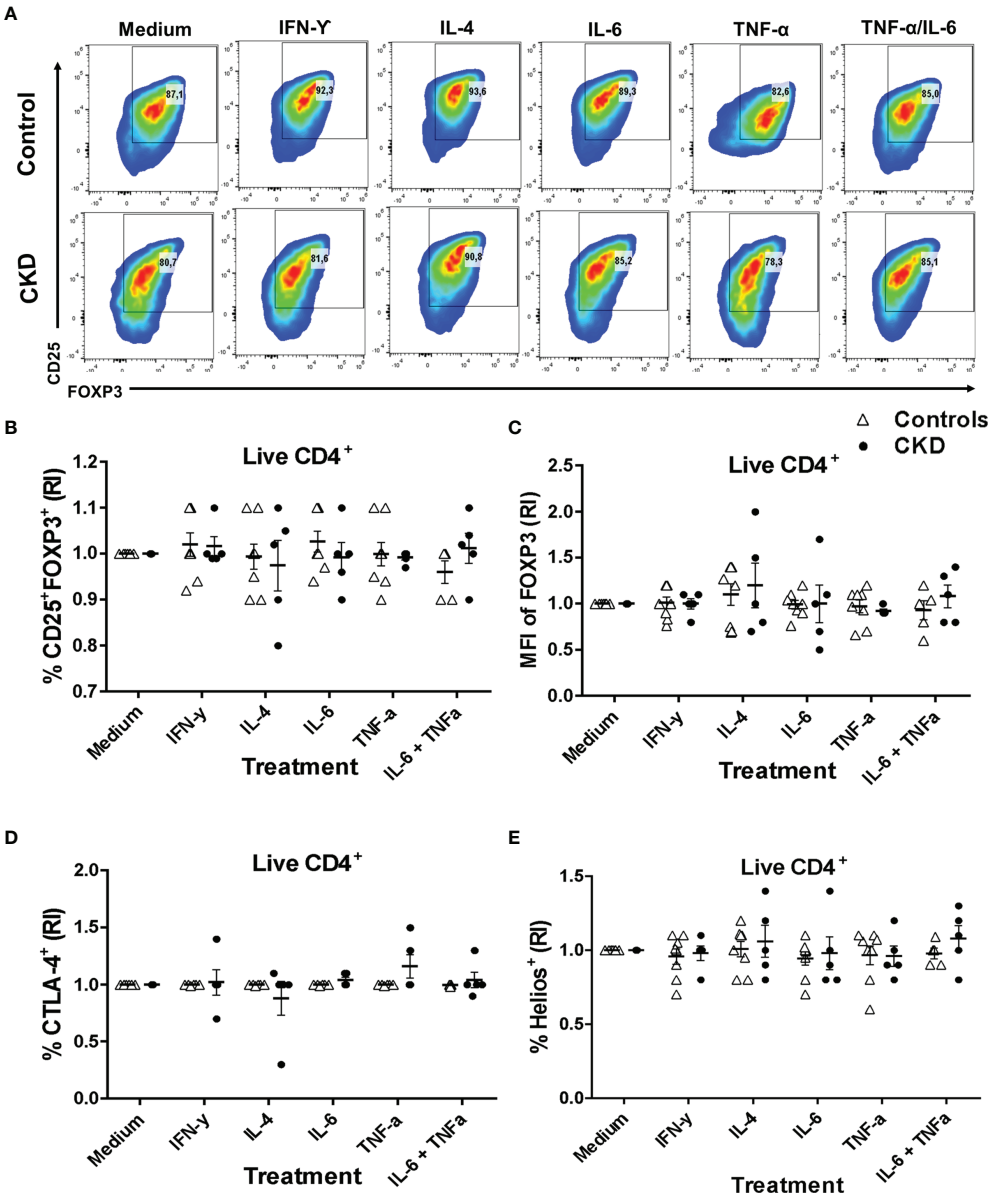

FIGURE 6 | Expanded alloantigen-specific regulatory T cells (Tregs) maintain their immunosuppressive phenotype after stimulation in an inflammatory microenvironment. Long-term expanded Tregs from healthy controls (*white triangles*, $n = 7$) or patients with chronic kidney disease (CKD) (*black circles*, $n = 5$) were stimulated with anti-CD3/anti-CD28 in the presence or absence of inflammatory cytokines (IFN-γ, IL-6, IL-4, or TNF-α) for two additional weeks. The expressions of Treg markers were determined by flow cytometry. **(A–D)** Activation of allospecific Tregs from CKD patients in the presence inflammatory cytokines did not have an effect on the percentages of $CD25^+FOXP3^+$ **(A, B)**, CTLA-4^+ **(D)**, and $Helios^+$ **(E)** cells and on the expression of FOXP3 **(C)** to the same extent as the Tregs from healthy controls. A representative experiment is shown in **(A)**. The relative increase (IR) of the percentage or median fluorescence intensity (MFI) was calculated by dividing the value in the presence of the cytokine by the value in the absence of cytokines (medium). The results are shown as the mean ± SEM. Statistical analysis was performed using the Kruskal–Wallis test. No significant differences were observed.

expanded Tn cells, while Helios was only significantly detected in ^{allo}Tregs (**Figure S7**). Furthermore, to discard that this phenotype was not a consequence of T-cell receptor activation, we evaluated the expressions of the Treg markers after a period of resting. These data support the notion that Treg identity is maintained in our long-term expanded cultures.

To further analyze the profile of our ^{allo}Tregs, we evaluated cytokine production, as previous studies have described the detection of inflammatory cytokines in expanded, non-purified allospecific Tregs (54). It was shown that our ^{allo}Tregs were unable to significantly release inflammatory cytokines. This discards the possibility of contamination of the activated

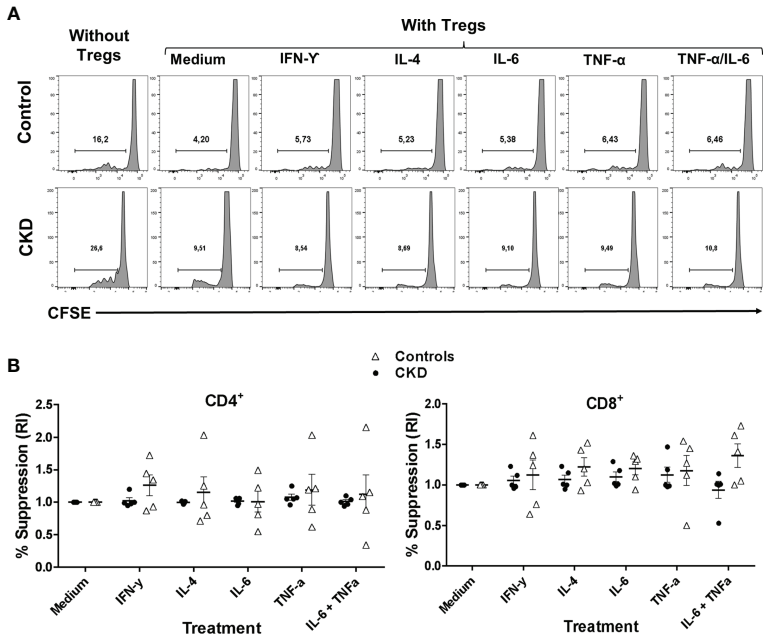

FIGURE 7 | Allospecific regulatory T cells (^{allo}Tregs) maintained their *in vitro* function after stimulation in an inflammatory microenvironment. Long-term expanded ^{allo}Tregs from healthy individuals (*white triangles*, $n = 5$) or patients with chronic kidney disease (CKD) (*black circles*, $n = 5$) were stimulated with anti-CD3/anti-CD28 in the presence or absence of inflammatory cytokines for two additional weeks, and then *in vitro* allospecific suppression assays were performed. **(A, B)** The ^{allo}Tregs from both patients and healthy controls maintained their capacity to suppress both $CD4^+$ (*left*) and $CD8^+$ (*right*) T-cell proliferation after expansion in the presence of all the inflammatory cytokines evaluated (IFN-γ, IL-6, IL-4, and TNF-α). A representative experiment is shown in **(A)**. The relative increase (IR) of the percentage of suppression was calculated by dividing the value in the presence of the cytokine by the value in the absence of cytokines (medium). All experiments were performed in duplicate. The results are shown as the mean ± SEM. Statistical analysis was performed using the Kruskal–Wallis test. No significant differences were observed.

Tconv or pro-inflammatory Treg conversion in our expanded cultures.

The expressions of immunoregulatory markers were in agreement with the efficient alloantigen-specific suppression of Tconv (>50%) using a 1:4 ratio (Treg/Tresp). This is in agreement with previous studies showing that the addition of rapamycin in cultures enhances the suppressive function of Tregs (38, 39). On the other hand, although the percentage of suppression achieved by our ^{allo}Tregs appeared to be lower than that previously reported, where the allospecific $CFSE^-$ Tregs were also purified (18), in this study, the expanded Tregs did not have a resting period prior to the suppression assay, and the authors performed the suppression assays based on using ^{3}H thymidine incorporation, which may have led to overestimation of the results (55). In another study, allospecific Tregs were able to effectively suppress responder T cells at a ratio of 1:100 (56); however, the authors used $CD4^+CD25^-$ sorted T lymphocytes instead of the $CD3^+$ T cells used in our experiments, and purified peripheral blood DCs were used instead of Mo-DCs. These differences make the suppression indexes less comparable.

Under inflammatory conditions, several studies have shown that IL-6, IL-4, IL-12, and TNF-α drive the loss of FOXP3 expression and, therefore, the suppressive capacity of Tregs (57–59). Alternatively, the exposure of Tregs to an inflammatory microenvironment may have promoted the co-expression of T helper (Th)-specific transcription factors that are key for Treg specialization and homing to inflammatory sites, including the allograft (23). Our data showed that ^{allo}Treg suppression under a pro-inflammatory milieu was similar to that in the absence of inflammatory cytokines (**Figure 4**). However, in these experiments, we cannot exclude the effect of cytokines on responder T cells, in addition to Tregs; this may explain the high heterogeneity of the responses observed among patients. Therefore, we also explored the effect of cytokines directly on Tregs after a 2-week activation period in the presence of the same stimuli. Interestingly, ^{allo}Tregs maintained high levels of FOXP3 and the characteristic markers, including those of Helios and CTLA-4 (**Figure 6**), which correlated with their suppressive function (**Figure 7**). Therefore, despite the long-term expansion of ^{allo}Tregs, they appeared to have maintained their phenotype and suppressive function under inflammatory conditions. Our study is in line with a previous report showing the effect of rapamycin on Treg stability through preventing the production of pro-inflammatory cytokines in expanded Tregs and inhibiting the conversion of Tregs toward an inflammatory phenotype (60). In addition, the expansion of allospecific nTregs in the presence of IL-2 alone was able to maintain >80% $FOXP3^+$ (**Figure S9D**), while both the induction and the maintenance of FOXP3 in expanded allospecific iTregs (22) and polyclonal iTregs (61) were shown

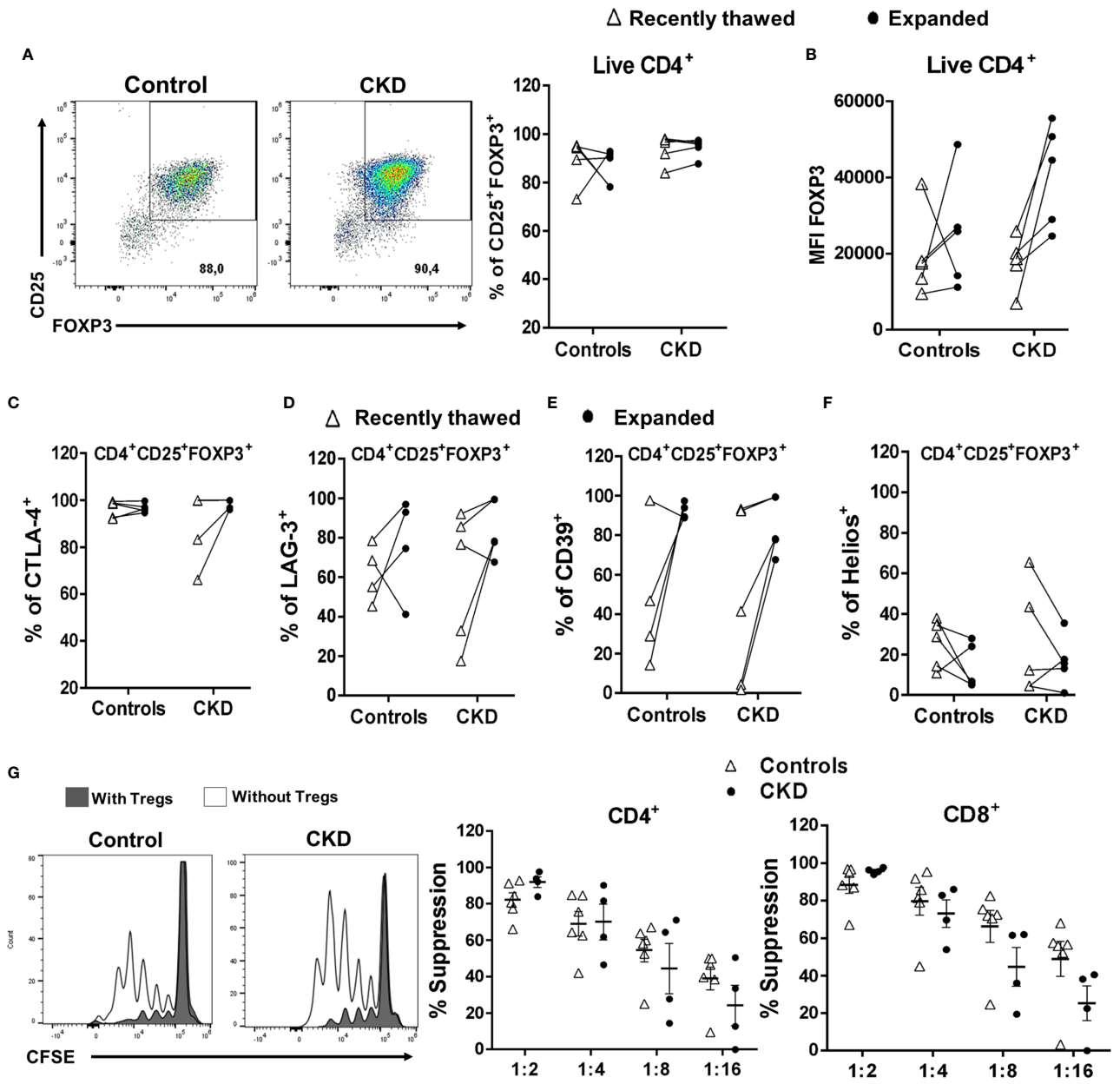

FIGURE 8 | Expanded regulatory T cells (Tregs) maintained their phenotype and *in vitro* function after long-term cryopreservation. On day 28 of polyclonal expansion, alloantigen-specific Tregs (^{allo}Tregs) from healthy individuals (*white triangles*, $n = 4$) or patients with chronic kidney disease (CKD) (*black circles*, $n = 4$) were cryopreserved for a long time (>6 months), and then the cells were thawed and polyclonally expanded. The expressions of the Tregs markers and their *in vitro* immunosuppressive function were determined by flow cytometry. **(A–F)** After 2 weeks of stimulation, thawed ^{allo}Tregs from both groups maintained the proportions of $CD25^+FOXP3^+$ **(A)**, CTLA-4^+ **(C)**, LAG-3^+ **(D)**, and $CD39^+$ **(E)** cells and the expression of FOXP3 **(B)**, but a low proportion of $Helios^+$ cells **(F)**. **(G)** Cryopreserved allospecific Tregs can suppress the proliferation of both polyclonal $CD4^+$ (*left*) and $CD8^+$ (*right*) T cells after 2 weeks of expansion. Representative experiments are shown in **(A, G)**. The results are shown as the mean ± SEM. Statistical analysis was performed using the Mann–Whitney *U* test or Wilcoxon test. No significant differences were observed.

to be highly dependent on the presence of TGF-β and rapamycin in the *in vitro* cultures.

The stability of FOXP3 expression has been shown to correlate with the complete demethylation of the TSDR within the *Foxp3* locus (31). The long-term expanded ^{allo}Tregs from both groups showed lower TSDR demethylation compared to that of freshly isolated Tregs. This is an unexpected result, as TGF-β has been linked to the epigenetic stabilization of FOXP3 expression through the inhibition of DNMT1 and Uhrf1 (40, 41). IL-2R signaling promoted the recruitment of TET2 (a

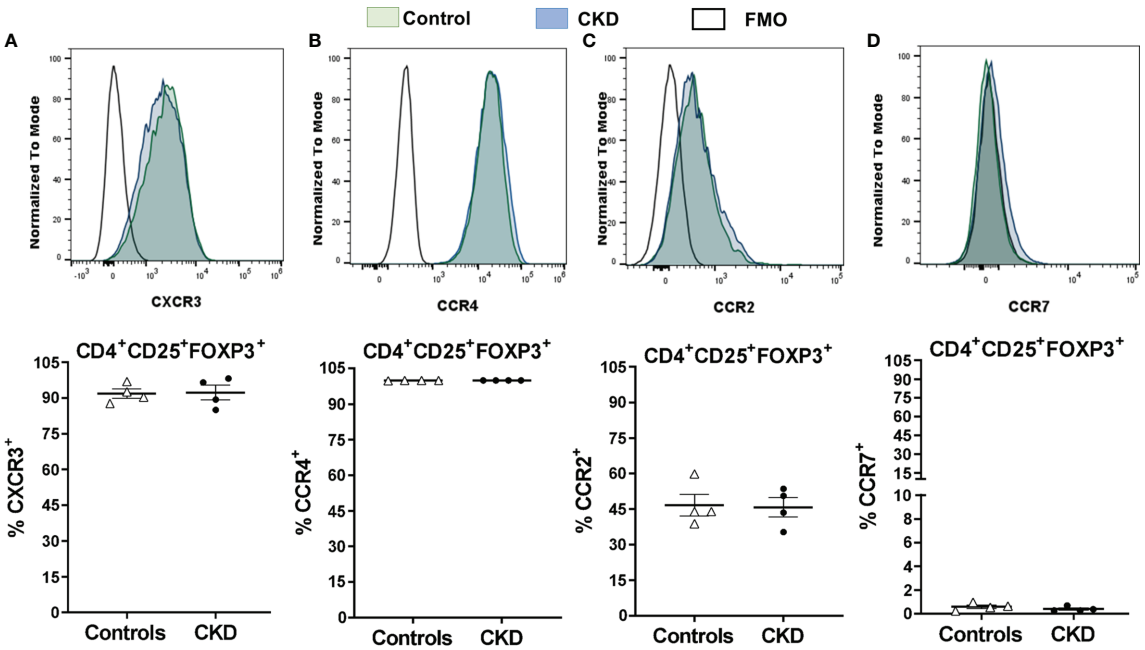

FIGURE 9 | Cryopreserved allospecific regulatory T cells (^{allo}Tregs) expressed high levels of the chemokine receptors CXCR3, CCR4, and CCR2. Long-term cryopreserved ^{allo}Tregs from healthy controls (*white triangles*, $n = 4$) or patients with chronic kidney disease (CKD) (*black circles*, $n = 4$) were thawed and polyclonally expanded for 2 weeks. The expressions of chemokine receptors were determined by flow cytometry. **(A, B)** The expanded Tregs of both groups had high percentages of CXCR3$^+$ **(A)**, CCR4$^+$ **(B)**, and CCR2$^+$ **(C)** cells, but a low proportion of CCR7$^+$ cells **(D)**. Representative experiments are shown in **(A–D)**. *White histograms* represent FMO (fluorescence minus one) controls. The results are shown as the mean ± SEM. Statistical analysis was performed using unpaired *t*-test. No significant differences were observed.

methylcytosine dioxygenase that catalyzes the demethylation of cytosines) to TSDR and maintained the demethylated CpG sites in the *Foxp3* locus (62, 63). Therefore, the increase in FOXP3 expression and suppressive function of the expanded ^{allo}Tregs did not correlate with the demethylation status of the *Foxp3* locus. Such discordance has been previously reported by our group in both expanded iTregs (22) and nTregs (21). Moreover, another study showed that the hypomethylation of TSDR in Tregs from juvenile idiopathic arthritis was not associated with their FOXP3 levels (64). Interestingly, it has been shown that targeted demethylation of *Foxp3*-TSDR does not ensure the stable suppressive function in FOXP3-induced primary T cells (65), indicating that TSDR demethylation by itself is not sufficient for Treg lineage commitment, although it still may be necessary for Treg stability.

The discrepancies observed between the demethylation of TSDR and the expression of FOXP3 might be explained by the fact that other potential epigenetic mechanisms or posttranslational modifications can regulate the identity of Tregs [revised in (66, 67)]. In this context, it has been shown that the loss of FOXP3 expression induced by the increase of CNS2 methylation in the *Foxp3* locus, observed under inflammatory conditions, can be counteracted by the recruitment of methyl-CpG binding protein 2 (MeCP2), an X-linked multifunctional epigenetic regulator, to *Foxp3*-CNS2, which in turn induces histone H3 acetylation, leading to stable FOXP3 expression (68). Additionally, the use of TGF-β in our cultures may have promoted the phosphorylation (69) and acetylation (70) of FOXP3 on multiple amino acid residues, thus reducing its ubiquitination and proteasomal degradation.

Additionally, as TSDR is still being used as the main marker for Treg stability for immunotherapy in the clinic, several approaches have been proposed to ensure the long-term functionality of Tregs *in vivo* for their effective use in immunotherapy. In this context, as an attempt to preserve *Foxp3* demethylation in the Treg cellular product used for immunotherapy, a recent report has demonstrated that a CD70$^-$CD27$^+$ population isolated from *in vitro* expanded polyclonal CD4$^+$CD25$^+$ Tregs gave rise to Tregs with a highly hypomethylated TSDR (71). In addition, the purification of long-term activated Tregs, based on CD137$^+$CD154$^-$ markers, allowed the *in vitro* expansion of Tregs with an epigenetic signature that is associated with functional stability (72).

Cryopreservation of Tregs offers several advantages for personalized immunotherapy, including their long-term storage and flexible timing and dosage of Treg infusion (8). However, whether this process affects the viability and/or stability of Tregs remains controversial. In this context, some reports have shown a reduction in the frequency of FOXP3$^+$ cells in cryopreserved PBMCs (73, 74), while other reports showed no significant differences between cryopreserved and freshly isolated Tregs (75). Similarly, previous studies (76, 77) have shown that

the expressions of the markers CD25 and FOXP3 in Tregs, as well as their suppressive function, were readily affected after thawing. Interestingly, the restimulation of these Tregs was able to restore their phenotype and function (76, 77). In the present work, we found that the cryopreservation of *in vitro* expanded ^{allo}Tregs preserved their FOXP3 expression immediately after thawing and, more importantly, that ^{allo}Tregs can be further expanded, reaching the numbers required for Treg adoptive cell therapy.

The chemokine receptor (CCR) expression profiles in Tregs may enhance the suppression of the alloreactive populations in order to establish efficient allograft tolerance (23). Our results showed that the cryopreserved ^{allo}Tregs expressed high levels of CCR4 and CXCR3 after two rounds of expansion, in agreement with a previous study (78) showing that both chemokine receptors were upregulated in Tregs stimulated in the presence of rapamycin. CXCR3$^+$ Tregs can efficiently restrict Th1 immune responses (79), while specialized memory CCR4$^+$ Tregs inhibited the Tconv proliferation by a FasL-dependent mechanism (80). Importantly, the role of infiltrating CXCR3$^+$IL-10$^+$TGF-β$^+$ Tregs was demonstrated in a kidney transplant mouse model, where the deletion of these cells led to allograft rejection (5). Finally, CCR2 expression in our expanded ^{allo}Tregs may be biologically relevant, as this receptor has been involved in Treg homing to both draining lymph nodes and allograft, thereby promoting the suppression of inflammatory T-cell responses, as demonstrated in an islet transplantation model (81).

In conclusion, we demonstrate that ^{allo}Tregs can be efficiently purified and expanded, maintaining a suppressive phenotype, most importantly from patients with CKD, who are candidates for kidney transplantation. The functionality shown after cryopreservation demonstrated the feasibility of the long-term storage of this cellular product and supports their potential use for personalized Treg therapy in transplanted patients. However, it is important to further investigate the epigenetic and posttranslational mechanisms underlying the FOXP3 expression and suppressive function maintained by our expanded ^{allo}Tregs and to explore whether recent protocols successfully used in the selection and expansion of polyclonal Tregs (71, 72) can be applied to ^{allo}Tregs in order to prove whether an epigenetic signature and/or phenotype can help identify the most appropriate Tregs for immunotherapy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee, Instituto Nacional de Ciencias Medicas y la Nutricion Salvador Zubiran (#1831). The patients/participants provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC-H performed the experiments, analyzed the data, and wrote the draft of the manuscript. EA-S, SA-C, KR-C, and NL performed the experiments. EA-S and SA-C wrote sections of the manuscript. JA contributed to the conception and design of the study. GS contributed to the conception and design of the study, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.686530/full#supplementary-material.

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Successful Milk Oral Immunotherapy Promotes Generation of Casein-Specific CD137$^+$ FOXP3$^+$ Regulatory T Cells Detectable in Peripheral Blood

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Background: Oral immunotherapy (OIT) is an emerging treatment for cow's milk protein (CMP) allergy in children. The mechanisms driving tolerance following OIT are not well understood. Regulatory T cells (T_{REG}) cells are key inhibitors of allergic responses and promoters of allergen-specific tolerance. In an exploratory study, we sought to detect induction of allergen-specific T_{REG} in a cohort of subjects undergoing OIT.

Methods: Pediatric patients with a history of allergic reaction to cow's milk and a positive Skin Pick Test (SPT) and/or CMP-specific IgE >0.35 kU, as well as a positive oral challenge to CMP underwent OIT with escalating doses of milk and were followed for up to 6 months. At specific milestones during the dose escalation and maintenance phases, casein-specific CD4$^+$ T cells were expanded from patient blood by culturing unfractionated PBMCs with casein *in vitro.* The CD4$^+$ T cell phenotypes were quantified by flow cytometry.

Results: Our culture system induced activated casein-specific FOXP3$^+$Helios$^+$ T_{REG} cells and FOXP3$^-$ T_{EFF} cells, discriminated by expression of CD137 (4-1BB) and CD154 (CD40L) respectively. The frequency of casein-specific T_{REG} cells increased significantly with escalating doses of milk during OIT while casein-specific T_{EFF} cell frequencies remained constant. Moreover, expanded casein-specific T_{REG} cells expressed higher levels of FOXP3 compared to polyclonal T_{REG} cells, suggesting a more robust T_{REG} phenotype. The induction of casein-specific T_{REG} cells increased with successful CMP desensitization and correlated with increased frequencies of casein-specific Th1 cells

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among OIT subjects. The level of casein-specific T_{REG} cells negatively correlated with the time required to reach the maintenance phase of desensitization.

Conclusions: Overall, effective CMP-OIT successfully promoted the expansion of casein-specific, functionally-stable FOXP3$^+$ T_{REG} cells while mitigating Th2 responses in children receiving OIT. Our exploratory study proposes that an *in vitro* T_{REG} response to casein may correlate with the time to reach maintenance in CMP-OIT.

Keywords: allergy, milk immunotherapy, regulatory T cells, clinical trial, tolerance, desensitization

INTRODUCTION

Cow's milk allergy (CMA) affects close to 0.6% of children under 2-years of age (1, 2). Up to 80% of children are expected to outgrow CMA by adulthood (3), but persistent CMA is a major risk factor for anaphylaxis due to accidental milk ingestion in school age-children (4). Cow's milk oral immunotherapy (CM-OIT) is emerging as an effective experimental approach to induce tolerance to milk protein, with up to 75% of patients successfully achieving desensitization (4–7).

However, there are still a number of patients who fail to achieve sustained unresponsiveness to CMP, lose their state of desensitization to CMP during the maintenance period or discontinue treatment despite the demonstrated clinical efficacy of CM-OIT (8). Furthermore, successful CM-OIT requires rigorous patient compliance, any deviation in protocol may prolong the length of time required to reach maintenance or increase the risk of developing an allergic reaction the scheduled CMP doses (9). Undoubtedly, individual differences in immunity can also contribute to the variable clinical outcomes observed in CM-OIT studies. Many efforts have been made to identify clinically relevant biomarkers that predict individual CM-OIT outcomes, none of which have been successful thus far (10, 11). Since the clinical response to CM-OIT is highly variable, developing biomarkers that successfully predict ability to achieve desensitization, time to reach maintenance or risk of developing adverse events during therapy would enable the individualization of CM-OIT and increase safety of the procedure.

Recently, investigators have focused on examining the upstream cellular mechanisms implicated in oral tolerance to food. Regulatory T cells (T_{REG}), a class of CD4$^+$ T cells expressing the transcription factor Forkhead box P3 (FOXP3), have been of particular interest given their key roles in induction and maintenance of peripheral tolerance to a plethora of self and non-self antigens (12). Allergen-specific T_{REG} cells can suppress both innate and adaptive arms of an allergic response, preventing mast cell activation, IL-4 production, Th2 cell development and IgE production by B cells (13).

T_{REG} cells can be readily measured in the peripheral blood and defects in their abundance and function have been implicated in the pathophysiology of food allergy (14). Indeed, mutations within the FOXP3 locus are associated with the development of severe food allergies due to a widespread loss of tolerance to innocuous antigens (15). Children with IgE-mediated food allergy have significantly lower FOXP3 expression compared to healthy controls (16, 17), and decreased frequencies in circulating T_{REG} cells after allergen exposure (18–20). In patients with peanut allergy, OIT increases both the abundance and suppressive function of T_{REG} cells as well as induces epigenetic changes such as hypomethylation of the FOXP3 locus required for maintenance of a stable suppressive T_{REG} cell phenotype (21). In children with milk allergy, those who tolerate baked milk have a higher frequency of peripheral blood casein-specific suppressive FOXP3$^+$CD25$^+$CD127$^-$ T_{REG} cells compared to children who do not, and this correlates with a higher likelihood of achieving milk tolerance (14). Similarly, children who outgrow their milk allergy have higher levels of peripheral CD4$^+$CD25$^+$ T_{REG} cells and lower *in vitro* T-cell proliferative responses to ß-lactoglobulin than those who do not (22). However, while the frequencies of antigen-specific T_{REG} cells and their secreted cytokines (IL-10, TGFβ) increase during OIT (23), neither successfully predict OIT outcomes (10).

In addition to potential disease heterogeneity and methodological variations that may have contributed to failed prediction of OIT outcomes in these studies, lack of reliable human T_{REG} cell markers is a significant limitation. T_{REG} cells are a functionally heterogenous population (24, 25) and traditional markers like CD25, CD127 and FOXP3 do not adequately discriminate between T_{REG} from T_{EFF} cells particularly in settings of T cell activation like allergy (25, 26). Most commonly used T_{REG} markers are also inducible on effector T cells (T_{EFF}) upon TCR-mediated activation, blurring the distinction between human T_{REG} and activated T_{EFF} cells, increasing the functional heterogeneity of the population and confounding the interpretation of results (25). Importantly, we have previously shown that expression of the transcription factor Helios alongside FOXP3, can reliably discriminate stably-suppressive T_{REG} cells from T_{EFF} cells in activated immune settings (25). Moreover, the differential expression of CD137 (4-1BB), a direct target of FOXP3, and CD154 (CD40 ligand) can further discriminate recently activated, functionally suppressive T_{REG} from activated T_{EFF} cells in human peripheral blood (27).

In this pilot CM-OIT clinical study, we performed in-depth, phenotypic characterization of CD4$^+$ T cell subsets specific to casein, the major protein allergens in cow's milk. We aimed to evaluate whether CM-OIT induced casein-specific, stably-suppressive FOXP3$^+$Helios$^+$ T_{REG} cells and whether this cellular response correlated with successful OIT. Here, we

characterized casein-specific T_{REG} and T_{EFF} cell phenotypes, based on differential CD137 (4-1BB) and CD154 (CD40L) expression, respectively, at several time-points during CM-OIT in 7 pediatric patients that successfully achieved CMP desensitization. We hypothesized that successful CM-OIT would require the expansion of casein-specific $CD137^+$ T_{REG} cells rather than the polyclonal expansion of total peripheral blood T_{REG}. Here, we propose that peripheral casein-specific $CD137^+$ T_{REG} responses during CM-OIT can be used to identify patients likely to achieve successful CMP desensitization and may correlate with CM-OIT time to reach maintenance.

MATERIAL AND METHODS

Human Subjects

Seven patients were recruited from a prospective randomized-controlled trial aiming to compare adverse events in patients undergoing CM-OIT to patients that continued to avoid CMP. This study was conducted at the Pediatric Allergy and Clinical Immunology Department of the Montreal Children's Hospital (MCH) in Montreal, Quebec, Canada (4). Informed consent was obtained for every patient and the study was approved by the Research Ethics Board of the McGill University Health Center (PED-12-090).

Whole blood samples were obtained from 7 children who successfully completed CM-OIT (defined as successful challenge to 200 ml milk or 8000 mg milk protein) and from one healthy non-allergic control for comparison (26-year-old male), depicted in **Figure 4**. Briefly, for each study patient, IgE-mediated CMA was diagnosed by compatible clinical history and positive skin prick testing (SPT) with commercial CMP extract (≥3 mm over saline control) or positive serum casein-specific IgE levels (>0.35k U/L). Placebo-controlled single-blinded oral challenge to CM was used to confirm CMP allergy, and patients were assigned in a 1:1 ratio to either CM-OIT or CM avoidance for 1 year with crossover at the end of this period. The CM-OIT protocol started with rush desensitization and was followed by an early escalation phase (E; dose escalation from 6 ml to 25 ml of CM), a late escalation phase (L; dose escalation from 125 ml to 200 ml of CM) and a maintenance phase (M; maintained 200 ml of CM) (illustrated in **Figure 1A**). Blood samples were taken before OIT (baseline or B), during the E phase, the L phase, and 6 months after reaching the M phase (4).

Peripheral Blood Mononuclear Cells and Lymphocyte Isolation

Whole blood samples were collected at B, E, L, M phase timepoints as well as from the healthy non-allergic control, as described above. PBMC were isolated from heparinized blood using Ficoll-based density gradient centrifugation. Isolated lymphocytes were labelled with CTV (Cell Trace Violet) or CFSE (carboxyfluorescein diacetate succinimidyl ester) and distributed into 96-well flat-bottom plates at a concentration of 5×10^5 cells/well. Casein was dissolved in sodium hydroxide for 12 hours and adjusted to a pH of 7.3-7.4 with HCl before use. Lymphocytes were incubated with prepared casein protein (500μg/ml) or medium alone (RPMI 1640 supplemented with 10% Nu-serum) and cultured at 37°C in a 5% CO_2 humidified incubator for 10 days, fresh media was replenished twice daily.

IgE and IgG Detection

Milk/casein-specific serum immunoglobulins were measured by ELISA. The 96-well polystyrene plates were coated with casein or capture antibodies for IgE or IgG4. Casein was dissolved using 1M NaOH for 4 hours. The protein concentration was adjusted with coating buffer to 20 ug/ml. Capture antibodies were diluted 1:3000 with coating buffer (pH 9.6). The coated plates were incubated overnight at 4°C. Coated plates were washed twice with PBS-T containing PBS (pH 6.8) and 0.05% Tween 20. The plates were blocked with 1% bovine serum albumin (BSA) in PBS-T for 2 hours at room temperature (RT), washed, and 50 ul of milk OIT participant serum diluted in blocking buffer was added to the plates and incubated for 2 hours at RT. Each participant serum sample was added in duplicate.

Serial dilutions of known concentrations of IgE or IgG4 standard were added to wells coated with IgE or IgG4 capture antibodies. Blank wells, wells containing only blocking buffer, and well containing serum from non-milk allergic healthy volunteers were used as negative controls. Following four washes with PBS-T, the plates were incubated for one hour at RT with biotinylated goat anti-human IgE antibody diluted 1:3000 or biotinylated mouse anti-human IgG4 antibody diluted 1:250 in blocking buffer. The plates were then washed twice with PBS-T then incubated for one hour at RT with Streptavidin-HRP. After four washes with PBS-T, 50ul of tetramethylbenzidine (TMB) was added to each well then incubated for 15 minutes at RT. The reaction was stopped with 50ul of 1M phosphoric acid. The optical density was measured at 450nm with a reference wavelength of 570nm. Values were converted from ng/mL to kU/L by dividing by a factor of 2.4.

Multi-Parametric Flow Cytometry

Lymphocytes were collected and stained with Viability dye (Fixable Viability Dye eFluor™ 780) and fluorescent monoclonal antibodies: anti-CD3-BV785 (clone OKT3), CD4-FITC (RPA-T4), CD8-PerCp-Cy5.5 (RPA-T8) and CD137-BV650 (4B4-1). Additional intracellular staining with anti-FOXP3-PE (206D), Helios-PE-Cy7 (22F6), CD154-APC (24–31) was performed after fixation/permeabilization of the cells using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Detection of intracellular cytokines was performed by stimulating lymphocytes with Phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) and ionomycin (1 μg/ml) (Sigma-Aldrich) in the presence of the Monensin-based Golgi inhibitor, Golgi Stop (BD Bioscience) for 3 h. Cells were stained using the same strategy as before, except CD4-AF700 (clone RPA-T4) was stained intracellularly following fixation/permeabilization. We evaluated cytokine production by staining with IL-4-PerCP-Cy5.5 (8D4-8) and IFN-γ-BV605 (B27) antibodies. Cells were acquired on a BD LSRFortessa X flow

cytometer (BD Bioscience) and analyzed using FlowJo version 10 software (FlowJo, LLC).

Statistical Analysis

A non-parametric one-way ANOVA followed by a Dunn's Multiple Comparison post-test was used for longitudinal comparisons of parameters across more than two phases of the study (SPT wheal size, casein-specific sIgE and sIgG levels, changes in the proportions of peripheral T_{REG} subsets), while a Wilcoxon Signed Rank test was used for longitudinal comparisons across two phases only (frequencies of peripheral Th1 and Th2 cells). To determine correlations between $CD137^+$ T_{REG} cells and cytokine-producing T_{EFF} cells or number of escalation days, we conducted a Pearson correlation. For comparisons of cell proportions or protein expression (MFI) between two or more T cell populations within a single phase of our study, a Wilcoxon Signed Rank was employed. Parametric unpaired student's t-test or two-way ANOVA with Tukey's post-testing were used to determine significance in *in vitro* experiments completed in triplicates from a single individual. A two-sided p-value of <0.05 was considered statistically significant. Statistical analyses were performed using Prism 7 Software (GraphPad, San Diego, CA).

RESULTS

Successful OIT Patients Show Decreased Cow's Milk SPT and Increased Casein-Specific IgG4 Responses

The details of the global trial design were recently published and is depicted in **Figure 1A** (4). Seven children from this cohort who successfully achieved CMP-OIT maintenance dosing were randomly selected for this study. Baseline demographics and clinical characteristics of all subjects are outlined in **Table 1**. The mean age was 12 years and 4/7 were female (57%). All patients reached the target maintenance dose of 200 ml with an average escalation period of 266 days (range: 168-504, IQR=98). The mean cow's milk SPT was 10.5 mm (range: 8-15, IQR=1.75) at study entry and 4.79 mm (range: 0.5-9, IQR=4) after 6 months of CM-OIT maintenance, representing a significant decrease from baseline (p=0.03) (**Figure 1B**). Casein-specific sIgE were available in all 7 patients but sIgG4 levels were only available for 6/7 patients. No significant changes in casein-specific IgE levels were detected during the study period (p=0.15) (**Figure 1C**), whereas casein-specific IgG4 increased in all patients by the M phase (p=0.0071) (**Figure 1D**). There was no

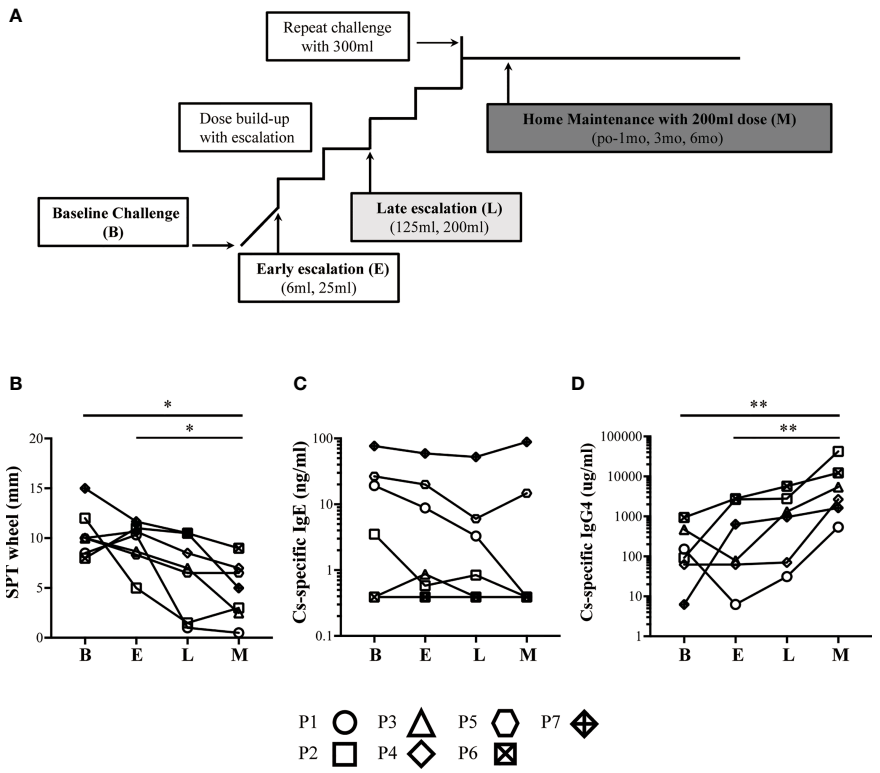

FIGURE 1 | Successful OIT patients have increased levels of casein-specific IgG4 and whole milk SPT responses. **(A)** Typical approach to cow's milk allergy immunotherapy. **(B)** SPT wheel size (mm) steadily decreased during CM-OIT in patients successfully achieving desensitization. **(C)** Casein-specific IgE (kU_A/L) levels in successful OIT patients at baseline **(B)** did not decrease significantly during the early escalation phase E, late escalation phase L or months after reaching maintenance M. **(D)** Casein-specific IgG4 (kU_A/L) steadily increased during CM-OIT in patients successfully achieving desensitization. Data is shown from 7 patients with each symbol representing a single patient. Casein-specific IgG4 levels were missing for P5. P-values were determined using a one-way ANOVA with a Dunn's Multiple Comparison post-test (*p < 0.05, **p < 0.01).

TABLE 1 | Baseline patient characteristics.

	Age (years) /Gender	Cumulative dose (ml)	Systemic allergy	SPT (mm)				Casein-specific IgE (ng/ml)				Casein-specific IgG4 (μg/ml)				Escalation days
				B	E	L	M	B	E	L	M	B	E	L	M	
P1	7/M	0.1	Asthma Eczema AR	8.5	10.33	1	0.5	19.23	8.81	3.29	0.39	151.33	6.25	31.25	538.1	196
P2	15/F	14.4	Asthma	12	5	1.5	3	3.53	0.58	0.84	0.39	92.42	2672.38	2790.75	42219.36	252
P3	12/M	89.4	Asthma	10	8.67	7	2.5	0.39	0.87	0.39	0.39	468.62	78.69	1320.29	5416.46	238
P4	14/F	89.4	Asthma Eczema	10	10. 67	8.5	7	0.39	0.39	0.39	0.39	62.5	62.5	70.20	2661.97	182
P5	12/F	44.4	Asthma Eczema	10	8.33	6.5	6.5	26.7	19.96	6.03	14.75					168
P6	12/F	1.4	Asthma Eczema	8	11	10.5	9	0.39	0.39	0.39	0.39	940.28	2779.05	5705.03	12276.90	504
P7	14/M	0.1	Asthma Eczema	15	11.67	10.5	5	76.77	59.2	52.19	88.63	6.25	633.38	964.94	1639.09	322

B, Baseline; E, Escalation; L, Late L; M, Maintenance.

correlation between SPT size, casein-specific IgE or IgG4 levels and individual time required to reach maintenance (data not shown).

Desensitization Is Associated With Casein-Specific T_{EFF} Cells With Altered Cytokine-Secreting Potentials

PBMC from each study subject was cultured with casein or Tetanus Toxoid (TT) for 10 days before T cell profiles were evaluated by flow cytometry. CM-OIT dose escalation was associated with the increased expansion of IFN-γ-producing Th1 ($CD4^+Foxp3^-$) cells following *in vitro* casein challenge (**Figures 2A, C**, P=0.0625). In contrast, IL-4-producing Th2 cell expansion following casein challenge tended to decrease during CM-OIT dose escalation (**Figures 2B, D**, P=0.0625). Correspondingly, the ratio of Th1 to Th2 cells increased between E and L phases (**Figure 2E**, P=0.0625), albeit not significant. Analysis of Th1 and Th2 cells were only completed on 5 patients during E and L phases due to sample availability. Our data demonstrates a deviation in circulating Th2 responses towards Th1 immunity over the course of CM-OIT.

Casein-Specific Expansion of Stably-Suppressive $FOXP3^+Helios^+$ T_{REG} Cells

To evaluate a potential increase in immunoregulation with CM-OIT, we aimed to characterize T_{REG} cells both *ex vivo* and in our *in vitro* casein re-stimulation system. We compared the phenotypic definition of T_{REG} cells using traditional markers ($CD25^{High}$ $CD127^{Low}$) to T_{REG} cells defined by FOXP3 and Helios co-expression in a representative CMA patient before and after reaching maintenance dosing (**Figures 3A, B**). Indeed, we have previously shown that $FOXP3^+$ $Helios^+$ T_{REG} cells represent a stably suppressive population of T_{REG} in healthy individuals (24, 25). *Ex vivo* and following *in vitro* stimulation with TT (antigen-specific T cell activation), the $CD25^{High}CD127^{Low}$ gating excluded more than half of the $FOXP3^+Helios^+$ T_{REG} cells (**Figures 3C, D**). In contrast, after αCD3 stimulation (strong polyclonal T cell activation), the $FOXP3^+Helios^+$ gating was more stringent than $CD25^{High}CD127^{Low}$ gating with the latter definition also including $FOXP3^-$ T_{EFF} cells and $FOXP3^+$ $Helios^-$ T_{REG} cells alongside $FOXP3^+Helios^+$ T_{REG} cells (**Figures 3C, D**). Thus, we decided to define T_{REG} cells as $FOXP3^+Helios^+$ in both CM-OIT and our *in vitro* culture systems.

In healthy, non-allergic control conditions, casein stimulation elicited a weak $FOXP3^+Helios^+$ T_{REG} proliferative response compared to stimulation with TT (**Figures 4A, B**). However, in subjects with CMA, stimulation with casein elicited a robust proliferative response in $FOXP3^+Helios^+$ T_{REG} cells (**Figure 4C**), suggesting the presence of casein-specific T_{REG} cells circulating in these patients.

Differential Expression of CD137 and CD154 Distinguish Casein-Specific T_{REG} Cells and T_{EFF} Cells, Respectively

Recently, it was suggested that CD137 and CD154 differential expression can identify antigen-specific T_{REG} and T_{EFF} cells in human PBMC, respectively (27, 28). Hence, to evaluate the presence of casein-specific T cells in our *in vitro* culture system, we utilized these markers. Proliferating T_{REG} cells were characterized by a significantly higher expression of CD137 than their non-proliferating counterparts (**Figures 4C, D**); similarly, proliferating T_{EFF} expressed higher levels of CD154 than non-proliferating T_{EFF} cells (**Figures 4C, E**). These results show that within all casein-specific T cells, CD137 expression is confined to proliferating T_{REG} cells whereas CD154 expression is confined to expanding T_{EFF} cells. $CD137^+$ is a marker of proliferating casein-specific T_{REG} cells, whereas $CD154^+$ is a marker of proliferating casein-specific T_{EFF} cells. We then evaluated the difference between $CD137^+$ T_{REG} and $CD137^-$ T_{REG} in terms of FOXP3 and Helios expression levels (**Figure 5**). While $CD137^+$ T_{REG} cells expressed higher levels of FOXP3 at each timepoint (E, L, M) (**Figures 5B, C**), Helios was differentially expressed between $CD137^+$ T_{REG} and $CD137^-$ T_{REG} at the L and M phase (**Figures 5D, E**).

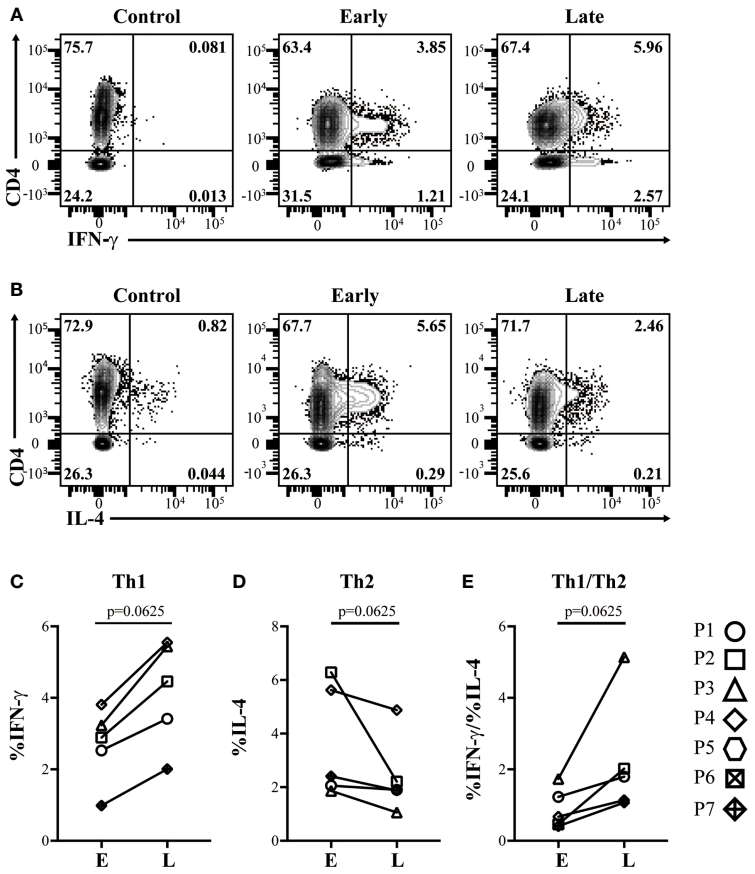

FIGURE 2 | Successful desensitization is characterized by expansion of IFN-γ-producing, but not IL-4-producing T_{EFF} cells following *in vitro* restimulation with casein. Representative flow cytometry plots from controls lacking PMA stimulation, early phase and late phase identifying **(A)**, $CD4^+$ IFN-γ^+ T_{EFF} cells, and **(B)** $CD4^+$ IL-4^+ T_{EFF} cells emerging in patient PBMC after a 10 day culture in the presence of casein. **(C)** Proportions of $CD4^+$ IFN-γ^+ T_{EFF} cells increased with dose escalation. **(D)** Proportions of $CD4^+$ IL-4^+ T_{EFF} cells from culture with casein decreased with dose escalation. **(E)** Ratios of $CD4^+$ IFN-γ^+ T_{EFF} to $CD4^+$ IL-4^+ T_{EFF} from culture with casein increased with dose escalation. Data is shown from 5 patients. P-values were determined using a Wilcoxon Signed Rank non-parametric test.

Induction of Casein-Specific $CD137^+$ T_{REG} Cells Correlates With Milk Sensitization, an Attenuated Th2 Response and Predicts the Length to Maintenance Phase

Since all patients successfully achieved the target CM-OIT maintenance dose, we sought to determine whether T_{REG} or T_{EFF} responses could be used as a marker of milk desensitization. Using the T_{REG} cell markers FOXP3 and Helios alone was insufficient to identify any differences in T_{REG} responses to *in vitro* casein challenge from PBMC isolated during E, L and M phases (**Figures 6A, B**). However, when stratifying T_{REG} cell responses based on CD137 expression, we observe that proliferating $FOXP3^+Helios^+CD137^+$ T_{REG} cells steadily increased during successful CM-OIT (**Figure 6C**). The proportion of $FOXP3^-Helios^-CD154^+$ T_{EFF} cells remained constant throughout the E, L and M phases (**Figure 6D**), suggesting that *in vitro* $CD137^+$ T_{REG} cell induction rather than a reduction in antigen specific $CD154^+$ T_{EFF} cell is associated with casein desensitization. Moreover, we found patients who reached maintenance phase under 36 weeks had highest frequency of $FOXP3^+Helios^+CD137^+$ T_{REG} than patients with more than 36 weeks to maintenance phase at M (**Figure 6C**), suggesting higher frequency of $FOXP3^+Helios^+CD137^+$ T_{REG} may be related to patients reaching M earlier. In early and late phases, the induction of $FOXP3^+Helios^+CD137^+$ T_{REG} cells correlated with an increase in the frequency of T_{EFF} cells with a Th1 phenotype and Th1/Th2 ratio *in vitro* (**Figures 6E, G**). There was also a modest negative correlation between $FOXP3^+Helios^+CD137^+$ T_{REG} and the frequency of T_{EFF} cells with a Th2 phenotype, albeit not significant (**Figure 6F**). Lastly, there is a negative correlation between the proportion of $FOXP3^+Helios^+CD137^+$ T_{REG} and the number of escalation days required to reach maintenance at E (**Figure 6H**), this is also observed for L and M, albeit non-significant (**Figures 6I–J**). This suggests that $FOXP3^+Helios^+CD137^+$ T_{REG} at E may correlate with individual time to reach maintenance.

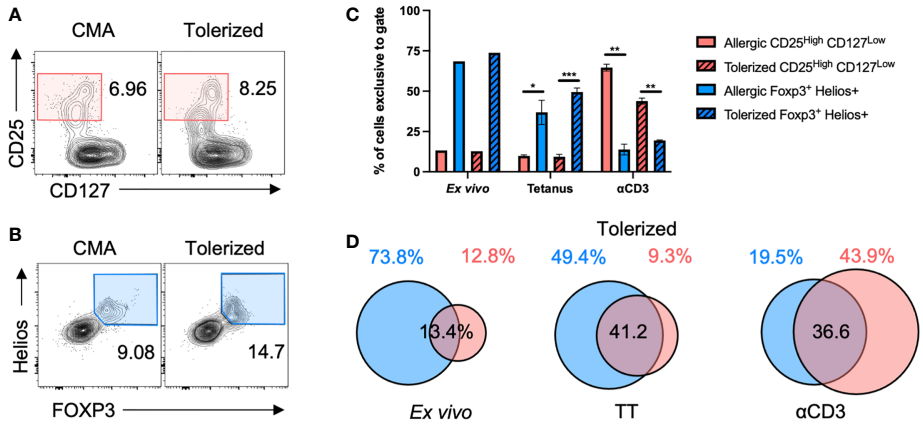

FIGURE 3 | FOXP3$^+$Helios$^+$ is a stringent definition for T_{REG} cells. PBMC from a representative CMA patient before and after tolerization were stimulated with TT or αCD3 for 4 days before staining for T_{REG} cells in flow cytometry. **(A)** Sample flow cytometry plots showing CD25^{High}CD127^{Low} T cells, and **(B)** FOXP3$^+$Helios$^+$ T_{REG} cells both pre-gated on CD4$^+$ T cells. **(C, D)** The proportion of CD4$^+$ cells captured by either CD25^{High}CD127^{Low} gating or FOXP3$^+$Helios$^+$ gating that were exclusive to either CD25^{High}CD127^{Low} or FOXP3$^+$Helios$^+$ gates were plotted in **(C)** with the degree of overlap between both populations shown in Euler-diagrams in **(D)** Cultures were completed in triplicates from a single patient's PBMC (N=3). P-values were determined using a two-way ANOVA with a Tukey's post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Bars represent the mean ± s.d.

DISCUSSION

Cow's milk OIT is an effective treatment for inducing oral tolerance in milk-sensitized individuals. However, its clinical applicability is limited by the inability to predict the probability of achieving successful desensitization or sustained unresponsiveness. In this exploratory proof-of-concept study, we suggest that stably-suppressive, casein-specific CD137$^+$ FOXP3$^+$Helios$^+$ T_{REG} may be a good candidate biomarker for identifying patients most likely to achieve successful CMP desensitization and be useful to predict time to reach maintenance in patients undergoing CM-OIT.

We characterized the immune parameters of 7 children with successful CM-OIT at several timepoints during treatment. We began by evaluating the standard published biomarkers, namely SPT to cow's milk, casein-specific sIgE levels, casein-specific sIgG4 levels, as well as peripheral casein-specific Th1 and Th2 cells. As expected, casein-specific sIgE levels remained relatively stable during the study period, cow's milk SPT size decreased and casein-specific sIgG4 levels increased with successful desensitization. Most patients maintained a positive SPT to cow's milk and casein-specific sIgE levels in the maintenance phase, demonstrating an ongoing potential for reactivity to CMP despite clinical induction of desensitization.

Since allergen-specific T cell subsets are emerging as a potential prognostic indicator of OIT outcomes, we then examined at casein-specific T_{EFF} and T_{REG} subsets at each phase of our study. To identify casein-specific T cells, we labelled PBMC with either CTV or CFSE proliferation dyes to identify expanding (CTV^{low} or CFSE^{low}) subsets upon exposure to casein. We observed an expansion of IFN-γ-producing T_{EFF} (Th1) cells from culture with casein, with a modest corresponding decrease in IL-4-producing T_{EFF} (Th2) cells between E and L phases, but this was not seen across the entire study period. This observation is in keeping with previous reports that CM-OIT induces a shift away from the predominant Th2 response to milk protein early during the desensitization process (3). Mechanisms of tolerance likely differ between dose escalation and maintenance phase which may explain why Th1 prominence only increased significantly during dose escalation in our study. Although T_{EFF} subsets may change during OIT, predictive thresholds, appropriate timing of sampling and robust correlations with clinical phenotypes are lacking, and further studies are required to validate their clinical usefulness (10). Of note, we did not find any correlation between T_{EFF} subtypes and the time to reach maintenance.

Induction of allergen-specific T_{REG} cells has classically been shown to be a later effect of OIT, and product of local differentiation of conventional T cells into allergen-specific T_{REG} cells following allergen exposure. These induced T_{REG} cells (iT_{REG}) are less stable than their thymic-derived natural T_{REG} (tT_{REG}) counterparts and have the potential to lose their suppressive phenotype under specific inflammatory contexts (29). Although the mechanisms of OIT mediating allergen tolerance have not been completely elucidated, stable T_{REG} induction seems to be central for the achievement and maintenance of CMP desensitization and loss of suppressive function or possible conversion of these cells to a Th2 cell phenotype could be associated with OIT failure (30). Previous studies have routinely evaluated T_{REG} in the clinic to predict OIT responses, but have been limited by the availability and choice of relevant surface markers to identify functional T_{REG} phenotypes (10). While both iT_{REG} and tT_{REG} cell subsets may be engaged in milk OIT, our results indicate that the emerging casein-specific T_{REG} cells express Helios, a transcription factor more frequently

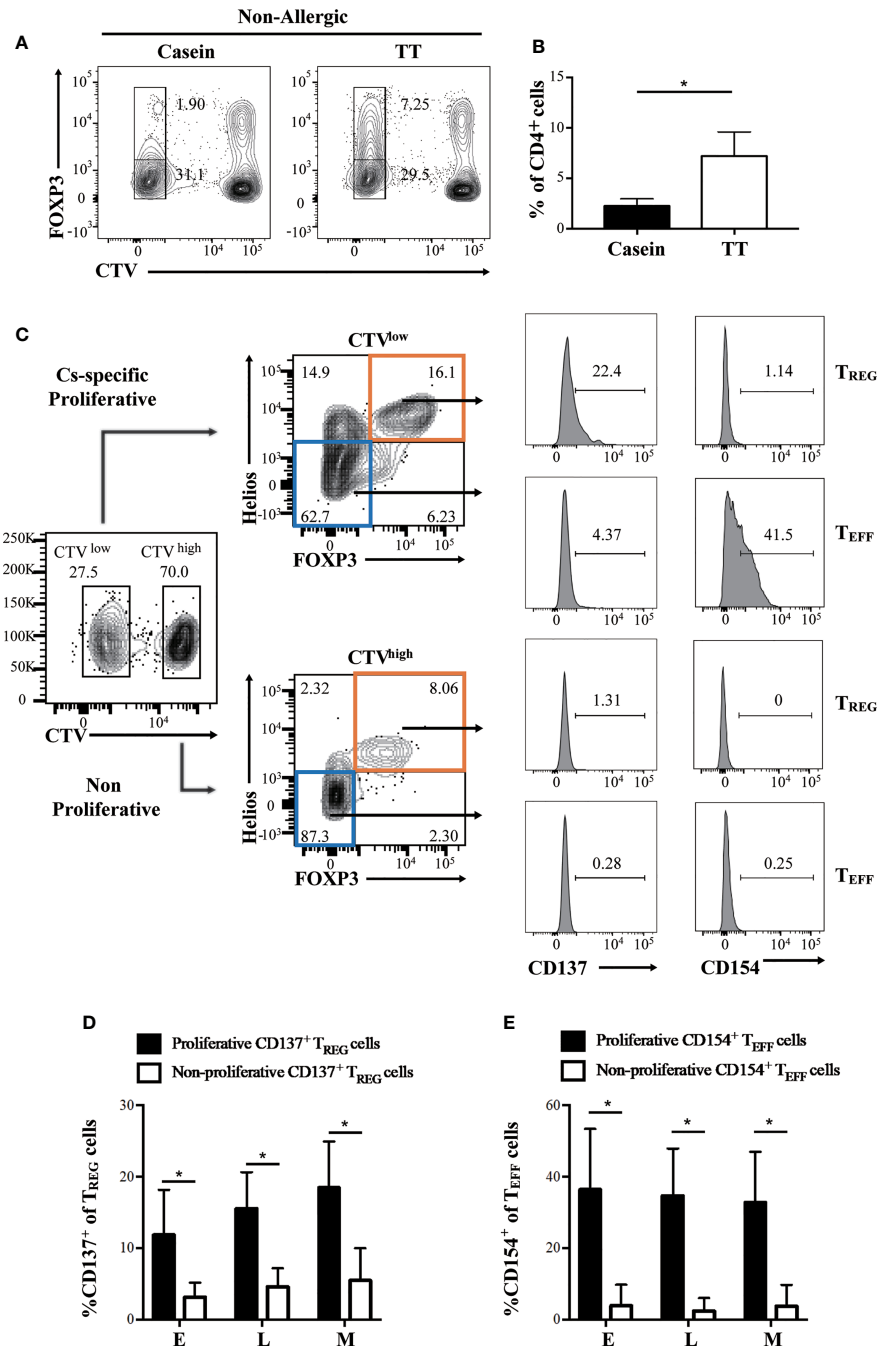

FIGURE 4 | CD137 and CD154 differentially identify casein-specific T_{REG} and casein-specific T_{EFF} cells. Proliferation of $CD4^+$ cells was assessed by flow cytometry-based CTV dilution analysis. **(A, B)** Healthy, non-allergic PBMC was cultured in the presence of casein or TT. **(A)** Representative flow cytometry plots of $FOXP3^+$ T cells depicting CTV dilution in $CD4^+$ T cells alongside **(B)**, the quantification (N=3). **(C–E)** Patient PBMC was cultured in the presence of casein for 10 days before evaluating expanded T cell responses by flow cytometry. **(C)** Flow cytometric gating strategy using a representative sample identifying proliferative (CTV^-, top panel) and non-proliferative (CTV^+, bottom panel) T_{REG} cells ($FOXP3^+Helios^+$) expressing CD137 and proportion of T_{EFF} ($FOXP3^-Helios^-$) expressing CD154 from a representative patient. **(D)** Expression of CD137 was significantly higher in proliferative $FOXP3^+Helios^+$ T_{REG} cells expanded in patient PBMC (N=3). **(E)** CD154 expression was significant higher in proliferative $FOXP3^-Helios^-$ T_{EFF} cells expanded in patient PBMC (N=3). The P-value in B was determined using unpaired t-test. P-values in **(C, E)** were determined using a Wilcoxon Signed Rank non-parametric test (*$p < 0.05$). Bars represent the mean ± s.d.

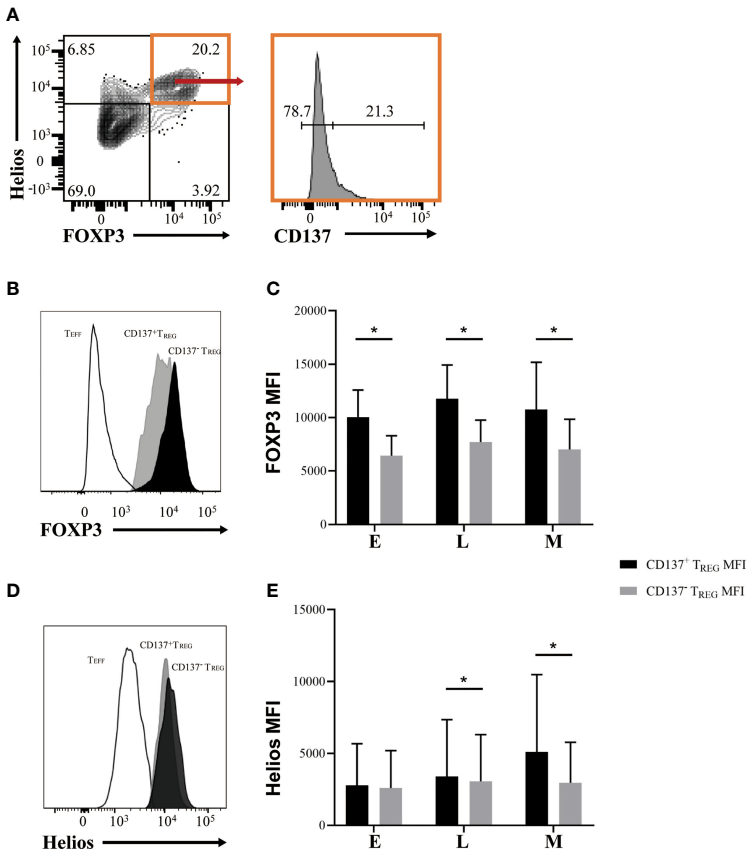

FIGURE 5 | Casein-specific CD137$^+$ T_{REG} cells express higher levels of FOXP3. **(A)** Representative flow cytometric plots identifying CD137$^+$ and CD137$^-$ T_{REG} (FOXP3$^+$ Helios$^+$) cells. **(B, C)** FOXP3 mean fluorescence intensity (MFI) is significantly higher in CD137$^+$ T_{REG} cells than in CD137$^-$ T_{REG} cells during all phases of CM-OIT (N=3). **(D, E)** Helios MFI is significantly higher in CD137$^+$ T_{REG} cells than in CD137$^-$ T_{REG} cells at L and M phases of CM-OIT (N=3). P-values were determined using a Wilcoxon Signed Rank non-parametric test (*P < 0.05). Bars represent the mean ± s.d.

associated with T_{REG} cells of thymic origin (tT_{REG}). Recently, however, Helios expression has also been shown to reflect T_{REG} stability and suppressive function, rather than mere T_{REG} lineage, as Helios acts to maintain the chromatin structure required for the induction and maintenance of the T_{REG} developmental program (31). Therefore, we interpret enhanced Helios expression as a marker of functionally suppressive T_{REG}.

CD4$^+$ T_{REG} cells have classically been defined by their expression of intracellular FOXP3, high cell surface expression of CD25 and low surface expression of CD127. However, CD25 and CD127 can be transiently modulated on CD4$^+$ T_{EFF} cells upon immune activation and FOXP3 can be transiently expressed in T_{EFF} cells upon T cell receptor (TCR) ligation (32, 33). Furthermore, although FOXP3 reliably identifies T_{REG} in their resting, non-activated state, not all CD25$^+$CD127^{low}FOXP3$^+$ T_{REG} clones are functionally suppressive (24). Thus, traditional markers of T_{REG} cells are not sufficient to identify functional and dysfunctional T_{REG} phenotypes.

Differential expression of a transcription factor of the Ikaros family, Helios, has been shown to reliably distinguish suppressive Helios$^+$FOXP3$^+$$T_{REG}$ from non-suppressive Helios$^-$FoxP3$^+$ T_{REG} clones (25). However, CTV^{low}CD4$^+$FOXP3$^+$Helios$^+$ T_{REG} did not vary significantly during early, late and maintenance phases of CM-OIT in our study indicating that Helios may not be sufficient to identify allergen-specific T_{REG}. Next, we sought to evaluate CD137 (4-1BB), a T_{REG} co-stimulatory receptor and a direct target of FOXP3 which has lately been identified as a robust marker of recently activated, antigen-specific, functionally suppressive iT_{REG} (27). Since effective T_{REG} suppression is antigen-specific, we hypothesized that successful CM-OIT would correlate with the expansion of casein-specific FOXP3$^+$Helios$^+$CD137$^+$ T_{REG} cells (CD137$^+$ T_{REG}) rather than polyclonal T_{REG} activation or decrease in allergen-specific T_{EFF}. In keeping with this hypothesis, we did observe that proliferating CD137$^+$ T_{REG} significantly increase during early, late and maintenance phases of CM-OIT. Moreover, we found that the induction of CD137$^+$ T_{REG} correlated with an increase in the frequency of T_{EFF} cells with a Th1 phenotype and a modest Th1/Th2 ratio suggesting that CD137$^+$ T_{REG} suppress Th2 immune responses in CM-OIT. The negative correlation between frequencies of CD137$^+$ T_{REG} cells and number of escalation days, and the finding that individuals with higher frequencies of

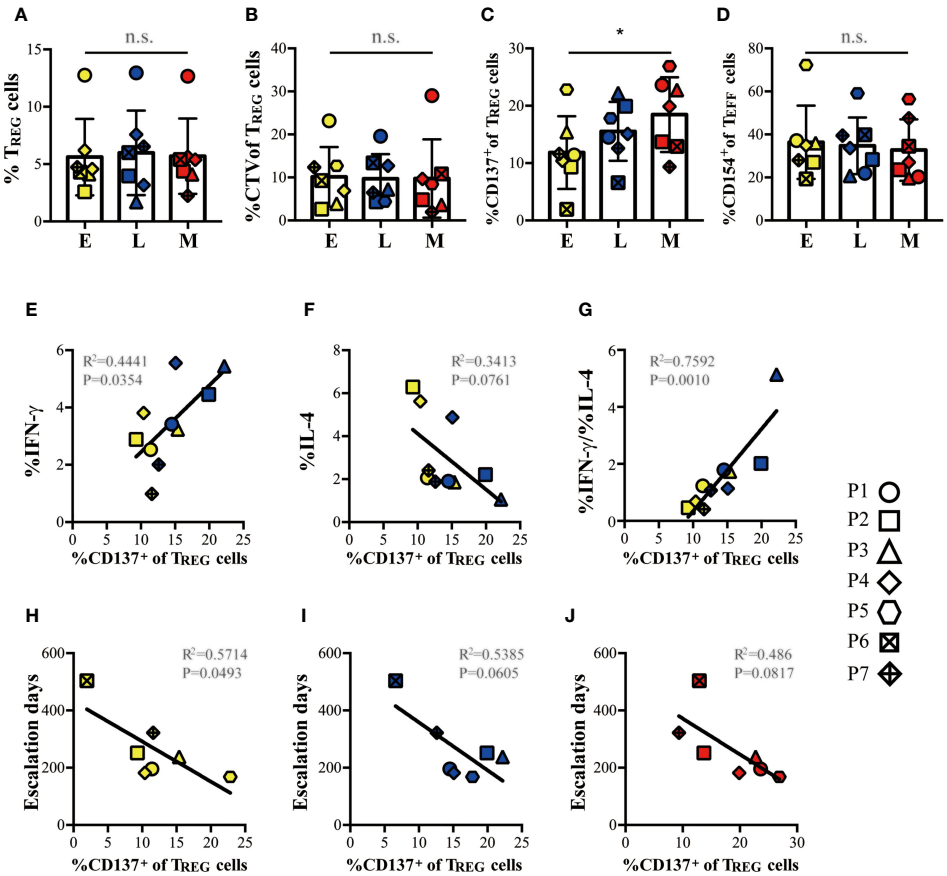

FIGURE 6 | Induction of casein-specific T_{REG} cells correlated with tolerance, suppressed Th2 responses, and with escalation days to maintenance. **(A)** Proportion of Helios$^+$FOXP3$^+$ T_{REG} cells and **(B)**, proportion of proliferative (CTV^{low}) Helios$^+$FOXP3$^+$ T_{REG} cells from total CD4$^+$ T cells expanded in our *in vitro* culture system with casein do not change significantly during E, L and M phases of CM-OIT. **(C)** When differentiating T_{REG} based on CD137 expression, we observe that casein-specific CD137$^+$ proliferative T_{REG} increase during Early, Late and Maintenance phase in successful CM-OIT patients. **(D)** There was no significant reduction in the proportions of CD154$^+$ proliferative T_{EFF} cells during CM-OIT. **(E, G)** The induction of CD137$^+$ proliferative T_{REG} correlated with an increase in the CD4$^+$IFN-γ$^+$ T_{EFF} cells from culture with casein and the ratio of CD4$^+$IFN-γ$^+$ T_{EFF} to CD4$^+$IL-4$^+$ T_{EFF} during Early and Late phase. **(F)** There was also a trend of correlation between CD137$^+$ proliferative T_{REG} and CD4$^+$ IL-4$^+$ T_{EFF} cells from culture with casein, although there is a no significance. **(H)** There is a negative correlation between the proportions of CD137$^+$ proliferative T_{REG} at **(E)** and escalation days to maintenance. **(I, J)** There was also a trend of correlation between the proportions of CD137$^+$ proliferative T_{REG} at Late and Maintenance phase. and escalation days to maintenance, albeit no significance. Each symbol represents 1 subject. Of 7 patients, 5 patients from E and L phase are involved in analysis/figure **(E–G)**. Yellow symbols represent data at Early phase Blue symbols represent data at Late phase. Red symbols represent data at Maintenance phase. P-values in **(A–D)** were determined using a one-way ANOVA with Dunn's multiple comparisons and in **(E–J)** with a Pearson correlation (*$p < 0.05$, n.s, not significant). Bars represent the mean ± s.d.

CD137$^+$ T_{REG} cells during the M phase needed less time to reach maintenance suggests that CD137$^+$ T_{REG} may be useful for predicting time to reach maintenance during CM-OIT. To ensure that casein tolerance was possibly driven by CD137$^+$ T_{REG} induction rather than a decrease in antigen-specific T_{EFF} cells, we compared proliferative T_{EFF} responses at each CM-OIT timepoint. Using CD154 as a marker of recently activated, antigen-specific T_{EFF} cells (27, 28), we found no significant difference in terms of proliferating CD4$^+$FOXP3$^-$Helios$^-$ CD154$^+$ T_{EFF} cells (CD154 T_{EFF}) throughout the study period.

Since a higher level of FOXP3 and Helios expression has been associated with increased suppressive potency and stability of the T_{REG} phenotype (25), we sought to determine differential expression of these two markers on CD137$^+$ and CD137$^-$ T_{REG} cells. Indeed, casein-specific CD137$^+$ T_{REG} cells exhibited a higher level of FOXP3 expression than their CD137$^-$ counterparts at each timepoint, whereas Helios was only differentially expressed between CD137$^+$ T_{REG} and CD137$^-$ T_{REG} at the M phase. These observations suggest that the circulating casein-specific CD137$^+$ T_{REG} cells acquire a stable and more suppressive phenotype throughout CM-OIT, and that Helios expression, thus far not described in the OIT literature, may be utilized as a marker of successful OIT.

In summary, we have performed an exploratory CM-OIT study and identified a potential clinically useful biomarker to identify patients most likely to achieve successful CMP tolerance and sustained unresponsiveness during CM-OIT. This remains a pilot study and our conclusions will be

validated in larger cohorts of patients which will include additional age appropriate non-allergic controls and patients having failed CM-OIT. The clinical utility of $CD137^+$ T_{REG} quantification during CM-OIT merits further investigation and validation in larger cohorts.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB of the McGill University Health Centre. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YZ, LL, GG, DK, SB, NP, DL, T-AA-A, and BT: sample processing, experimental design, assay development and execution, data analysis/reporting, and/or figure/manuscript preparation MB, BM, and CP: trial design, experimental design, data analysis and reporting, figure preparation, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Interleukin-5 (IL-5) Therapy Prevents Allograft Rejection by Promoting CD4$^+$CD25$^+$ Ts2 Regulatory Cells That Are Antigen-Specific and Express IL-5 Receptor

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CD4$^+$CD25$^+$Foxp3$^+$T cell population is heterogenous and contains three major sub-groups. First, thymus derived T regulatory cells (tTreg) that are naïve/resting. Second, activated/memory Treg that are produced by activation of tTreg by antigen and cytokines. Third, effector lineage CD4$^+$CD25$^+$T cells generated from CD4$^+$CD25$^-$ T cells' activation by antigen to transiently express CD25 and Foxp3. We have shown that freshly isolated CD4$^+$CD25$^+$T cells are activated by specific alloantigen and IL-4, not IL-2, to Ts2 cells that express the IL-5 receptor alpha. Ts2 cells are more potent than naïve/resting tTreg in suppressing specific alloimmunity. Here, we showed rIL-5 promoted further activation of Ts2 cells to Th2-like Treg, that expressed *foxp3, irf4, gata3* and *il5. In vivo*, we studied the effects of rIL-5 treatment on Lewis heart allograft survival in F344 rats. Host CD4$^+$CD25$^+$T cells were assessed by FACS, in mixed lymphocyte culture and by RT-PCR to examine mRNA of Ts2 or Th2-like Treg markers. rIL-5 treatment given 7 days after transplantation reduced the severity of rejection and all grafts survived ≥60d whereas sham treated rats fully rejected by day 31 ($p<0.01$). Treatment with anti-CD25 or anti-IL-4 monoclonal antibody abolished the benefits of treatment with rIL-5 and accelerated rejection. After 10d treatment with rIL-5, hosts' CD4$^+$CD25$^+$ cells expressed more *Il5ra* and responded to specific donor Lewis but not self. Enriched CD4$^+$CD25$^+$ cells from rIL-5 treated rats with allografts surviving >60 days proliferated to specific donor only when rIL-5 was present and did not proliferate to self or third party. These cells had more mRNA for molecules expressed by Th2-like Treg including *Irf4, gata3* and *Il5.* These findings were consistent with IL-5 treatment preventing rejection by activation of Ts2 cells and Th2-like Treg.

Keywords: interleukin-5, transplant tolerance, T regulatory cells, cytokines, allograft rejection, chronic rejection, CD4 + CD25 + Treg cells, Th2 cytokines

Abbreviations: BSA, bovine serum albumin; HRP, horse radish peroxidase; Ipi, intra-peritoneal injection; IL-, interleukin; mAb, monoclonal antibody; PBS, phosphate buffered saline; r, recombinant; Treg, T regulatory cells.

INTRODUCTION

With current immunosuppression, organ allografts are rarely lost from acute rejection but later rejection remains a major problem (1) in all forms of organ transplantation. No current therapy is effective at its prevention or treatment (2–4). Induction of alloantigen specific tolerance is a potential therapy to prolong graft survival.

Rejection is a complex immunological process, starting with $CD4^+$T cell activation by donor alloantigen (4, 5) resulting in a mononuclear cell infiltrate, T cell mediated injury (6, 7) and antibody deposition (8) with activation of complement (9). This leads to slow destruction of the allograft from vascular injury (10), destruction of the microcirculation (11, 12) and fibrosis (1).

The most frequently studied Treg are naïve/resting thymus derived $CD4^+CD25^+Foxp3^+$T cells (tTreg) (13, 14) however these alone do not mediate transplant tolerance. Transplanted tissues, while activating rejection responses, also induce alloantigen-specific $CD4^+CD25^+Foxp3^+$Treg (15–17).

$CD4^+CD25^+Foxp3^+$T cell population is heterogenous, containing three major sub-groups, as described by Miyara et al. (18). Understanding this heterogeneity may be useful in activating Treg as therapy (19–21), an approach that is yet to fully evolve, as reviewed (22). In **Table 1** we define the subsets of $CD4^+CD25^+Foxp3^+$T cells, relevant to the understanding of this work.

Within peripheral $CD4^+CD25^+$ cells in addition to naïve/resting tTreg there are tTreg that have been activated by antigen and cytokines known as activated/memory Treg, and effector lineage $CD4^+CD25^+$T cells that have been activated in periphery by antigen and transiently express CD25 and Foxp3, also known as pTreg/iTreg. Thus, the enriched $CD4^+CD25^+$ cells we study contain all three populations and have a vast array of T cell receptors that can each react to a specific antigen.

In animals that develop transplant tolerance, control of rejection is mainly mediated by alloantigen-specific $CD4^+CD25^+Foxp3^+$Treg (15–17, 23). In these animals, antigen-specific Treg are expanded. Thus, promotion of alloantigen-specific Treg could control rejection and establish operational tolerance (24).

Treg, either freshly isolated or after polyclonal expansion, need to be at ratios of ≥1:1 to effector T cells to fully suppress immune responses (14, 25) including allograft rejection *in vivo* (17) and proliferation *in vitro* of naïve $CD4^+$T cells to alloantigen in mixed lymphocyte culture (MLC) (26). However, *in vivo* the ratio of Treg ($CD4^+CD25^+Foxp3^+$T cells) to effector T cells ($CD4^+CD25^-Foxp3^-$) is highly regulated to ≤1:10 and ratios of 1:1 cannot be maintained.

The $CD4^+CD25^+Foxp3^+$Treg that are antigen activated and mediate alloantigen-specific tolerance are also present within the $CD4^+CD25^+$T cell pool. They are more potent at suppression, and have different properties and phenotypes to naïve/resting $CD4^+CD25^+Foxp3^+$Treg (27). Preparations of $CD4^+CD25^+$cells contain both naïve resting tTreg and activated antigen-specific Treg.

The precise pathway for activation of alloantigen specific $CD4^+CD25^+Foxp3^+$Treg are still not known, however. We have previously shown activation of naïve/resting Treg with specific-alloantigen and the Type-1 cytokine IL-2 induces a population of more potent antigen-specific Treg that express *Ifngr* and *Il12rb2* (28, 29). We have called these cells Ts1. Ts1 cells are promoted by alloantigen and the Th1 cytokines IFN-γ and/or IL-12 to Th1-like Treg that suppress at very low ratios (23) and can induce transplant tolerance.

In a rejection response, there is also activation of Th2, Th17 and other cell types that produce different cytokines to Th1 cells. These different cytokines in presence of alloantigen also promote activation of naïve Treg by separate pathways.

Relevant to this study, we described a second pathway of tTreg activation by Type-2 cytokines (28, 29) (**Figure 1**). Activation of tTreg by IL-4 a Type 2 cytokines is independent of IL-2, as tTreg express the IL4Rα. tTreg cultured with recombinant (r) IL-4 and alloantigen develop into more potent activated Treg that prevent allograft rejection mediated by naïve $CD4^+$T cells at a ratio of 1:10 and suppress specific anti-donor responses in MLC at ratios of 1:32 to effector $CD4^+CD25^-$ cells (29). In contrast, fresh naive $CD4^+CD25^+$ cells only fully suppress allograft rejection or anti-donor responses in MLC at 1:1 (26, 31). We called these IL-4 and antigen activated Treg, Ts2 cells (29). Ts2 cells express IL-5Rα, the specific-receptor for the Type 2 cytokine IL-5 (29), and are activated by IL-5 in the presence of specific antigen (30, 32). Using cells from animals tolerant to an allograft, we have shown that IL-5 promotes

TABLE 1 | Definition of subpopulations of cells within peripheral lymphoid $CD4^+CD25^+Foxp3^+$T cells relevant to this study.

Thymic derived naive/resting Treg.	Thymus derived $CD4^+CD25^+Foxp3^+$T cells that have not been activated by antigen since leaving the thymus. Known as tTreg or nTreg. These cells are the majority of $CD4^+CD25^+$T cells in naive animals.
Activated/ memory Treg	Thymus derived $CD4^+CD25^+$ $Foxp3^+$Treg that are activated by antigen in the periphery in the presence of cytokines which induces higher expression of CD25 and Foxp3 than in tTreg
Peripheral/ induced Treg	Effector lineage $CD4^+CD25^-Foxp3^-$T cells that have been activated by specific antigen in the absence of inflamatory cytokines such as IL-6 and IL-1 and transiently express CD25 and Foxp3. Known as pTreg or iTreg
Ts1 cells	tTreg that have been activated by a specific antigen and the Type-1 cytokine IL-2. Express receptors for Type-1 cytokines IFNGR and IL-12Rβ2. Ts1 cells are 10-64 times more potent than tTreg at suppression of responses to specific-antigen.
Th1-like Treg	Ts1 cells that have been activated by a specific-antigen and the Type-1 cytokines such as IL-12 or IFN-γ. IL-2 blocks induction of Th1-like Treg. Also express Th1 associated molecules IFN-γ, Tbet, CXCR3. Th1-like Treg's suppression is 100-1000 fold more potent than tTreg.
Ts2 cells	tTreg that have been activated by a specific antigen and the Type-2 cytokine IL-4. IL-2 is not required for induction of Ts2 cells as tTreg express IL-4Rα. Ts2 cells express receptors for Type-2 cytokine IL-5 and 10-32 times more potent at suppression of responses to specific antigen than tTreg.
Th2-like Treg	Ts2 cells that have been activated by a specific-antigen and the Type-2 cytokine IL-5. Also express Th2 associated molecules IL-5, GATA3, IRF4 and CCR8. Suppression 100-1000 fold more potent than tTreg

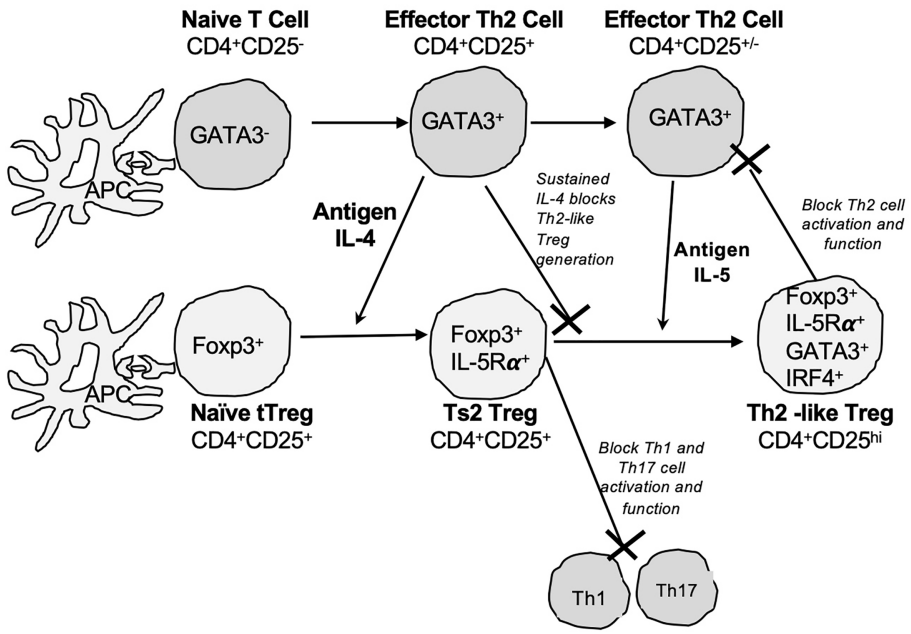

FIGURE 1 | Pathways for activation of naïve $CD4^+CD25^+Foxp3^+$Treg by Type-2 cytokines and alloantigen. We propose activation of naïve/resting thymic $CD4^+CD25^+$ Treg (tTreg) is driven by cytokines produced by activated effector T cells. The Type-2 cytokine and alloantigen activation pathway of tTreg parallels the activation of effector Th2 cells. Our hypothesis is based on the physiology of immune response. IL-4 is only produced by Th2 cells early in an immune response and late in the response IL-4 is replaced by other Th2 cytokines including IL-5 and IL-13. Our proposed model is that naïve T cells in the rejection response are activated to Th2 cells as well as Th1 cells. Th2 cells express transcription factor GATA-3 and produce Th2 cytokine IL-4 (Top row) in early stages of immune response. This IL-4 activates other naïve T cells to expand the immune response. In parallel, IL-4 also activates naïve/resting $CD4^+CD25^+Foxp3^+$Treg (tTreg) in a polyclonal fashion and this activation does not require IL-2. tTreg express IL-4Rα and can recognize graft alloantigen in the presence of IL-4 get activated to alloantigen-specific Treg that are induced to express IL-5Rα, the specific receptor for IL-5 (Bottom row). We call these activated antigen-specific $CD4^+CD25^+Foxp3^+$Treg Ts2 cells (29, 30). Ts2 cells diminish the immune response by inhibition of Th1 and Th17 cells promoting polarization to a Type 2 effector response (30). A sustained Th2 response results in production of IL-5 from Th2 cells ($GATA\text{-}3^+$), and diminished IL-4 production. In the second step of activation is IL-5 in presence of specific stimulating alloantigen promoting expansion of Ts2 ($IL\text{-}5R\alpha^+Foxp3^+$) to Th2-like Treg. Th2-like Treg express mRNA for *Foxp3, Gata-3*, *Irf4* and *Il5*. Th2-like Treg do not express key markers of Th1-like Treg such as *tbet*, *Ifng. Ifngr or Il12rb2*. The presence of IL-4 during late stage of immune response inhibits induction of Th2-like Treg.

survival of tolerance-transferring $CD4^+$T cells (33) and proliferation of $CD4^+CD25^+$T cells to specific alloantigen (31).

In rats, treatment with rIL-5 reverses autoimmunity (30, 32) and delays neonatal heart graft rejection (34), with inhibition of Th1 and Th17 while sparing of Th2 responses (30, 34). In autoimmunity, the immunosuppressive effect of rIL-5 requires host $CD25^+$T cells and IL-4 (30). rIL-5 therapy expands auto-antigen-specific Ts2 cells (30).

In this study we found re-culture of Ts2 cells with specific-antigen and rIL-5, in the absence of rIL-4, induced Th2-like Treg that expressed mRNA for *Gata*-3, Interferon regulatory factor 4 (*Irf*4), II-5Rα and the Th2 cytokine II-5. GATA-3 is the Th2 transcription factor. IRF4 is a transcription factor that is induced by TCR binding to antigen and promotes induction of Th2 cells but not Th1 responses (35). $CD4^+CD25^+Foxp3^+$ Treg that are activated by Type-2 cytokines (36) depend upon IRF4 to control effector Th2 responses (37).

We hypothesized that rejection responses would activate Th2 cells that produce IL-4 that together with alloantigen, would activate antigen-specific Ts2 cells. Treatment with rIL-5 early post-transplant, in the presence of alloantigeneic stimulation, could promote expansion of these alloantigen specific Ts2 cells and induction of Th2-like Treg. Such Th2-like Treg could complement induction of tolerance by Type-1 cytokine activated tTreg that may occur in parallel as described earlier.

We used Lewis heterotopic heart grafts in F344 hosts (38, 39) where rejection is slow as there is only one class I MHC incompatibility and no class II MHC incompatibilities. We found that treatment with rIL-5 prevented progression of rejection and induced prolonged allograft survival. Monoclonal antibody (mAb) treatment to deplete host $CD25^+$ cells or block host IL-4 impaired the rIL-5 effect. Host $CD4^+CD25^+$T cells had specificity for donor antigen when cultured with rIL-5 and expressed molecules associated with Ts2 and Th2-like Treg.

MATERIALS AND METHODS

Animals

F344 ($RT1^{lvl}$) rats were purchased from the Animal Resource Centre (Murdoch, WA, Australia). Lewis ($RT\text{-}1^l$), PVG ($RT1^c$) and DA(RT-1a) rats were bred and maintained in the animal house, Liverpool Hospital. All animals were fed standard chow and given water *ad libitum*. The housing and experiments were

in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and approved by the Animal Ethics Committee of the UNSW Sydney. Rats that received standard care in the animal house and not given any treatment or alloantigen were considered naïve.

Heterotopic Heart Graft Procedures

F344 male rats of 200g or more were anesthetized with isoflurane and grafted with heterotopic adult Lewis hearts from 180-230g donors, as described (40). Graft function was monitored daily during the treatment period then two to three times per week. Graft function was scored as *4.* for a strong and fast beat similar to an isograft, *3.* for mild graft swelling and slowing of graft contraction, *2.* for moderate swelling and slowing of graft heartbeat, *1.* for marked swelling and slowing of contraction, *0.5.* for marked bradycardia and minimal and variable contraction, and *0.* if no beat was detected. Total rejection was defined as a score of *0.5* or *0* observed for 10 days. In some tolerance models, graft function can improve days after what appears to be complete rejection. Thus, we observed graft function for weeks after major rejection. Some animals were sacrificed at the end of rIL-5 treatment for histology, as described (17).

Cytokines

Rat rIL-5 and rat rIL-4 were produced as supernatant from a transfected CHO-K1 cell line that was cultured in serum free medium and activity assessed in bioassays as described (41, 42). Supernatant was concentrated and rIL-5 quantified in a bioassay using the IL-5 dependent cell line B13 (a gift of Dr C. Sanderson, Curtin University, Perth WA, Australia), as described (43–46). 5000 Units of rIL-5 in 0.5 ml was given ipi as a daily dose. 5000 units of rIL-5 per day is well tolerated by rats, induces Ts2 cells to reverse autoimmunity and induces eosinophilia (22).

Treatment With Monoclonal Antibodies

To deplete $CD25^+$cells, the mAb NDS61 (gift of M Dallman, Imperial College London, UK) was given ipi at 7mg/kg daily from day 3 to 17 post-transplantation (30, 47). To block IL-4, 7mg/kg MRCOX81 (gift of N Barclay, Sir William Dunn School of Pathology, Oxford, UK) was given ipi on days 3-8 post-grafting, then every second day until day 15, as described (30, 41). These mAb were produced as described (15).

Experimental Plan for Transplant Experiments

Five groups of F344 rats with heterotopic Lewis heart grafts (n=4-5) were studied and animals were monitored for heart allograft function. A sham treated group received saline injections daily from Day 7-16 post-transplant and four groups were given rIL-5 daily for 10 days from day 7 to 16 post-transplant. One of these four groups, the short-term rIL-5 treated, had rIL-5 therapy stopped after day 16, this group was repeated three times with results of all animals combined (n=12). Another rIL-5 treatment group, the long term treated group, had rIL-5 therapy continued as three times a week after the day 16. For the remaining two groups that received rIL-5, one was also treated with anti-CD25 mAb and the other with anti-IL-4 mAb, as described above. Some animals were used for histology of the heart graft and/or collection of spleen and lymph node cells for enrichment of $CD4^+CD25^+$T cells for MLC. At the end of experiments, at about 60 days post-transplant, all graft recipients in groups 1, 2 and 3 were sacrificed for FACS, RT-PCR and MLC studies on enriched $CD4^+CD25^+$T cells.

Histology

Donor heart sections were paraffin fixed and stained with hematoxylin and eosin. The histology images shown in **Figure 5B** were taken by a Leica DFC 450C camera with 20x magnification on a Leica DM 2000 LED microscope as we have described in **Figure 5B** legend. For quantification of areas of myocyte necrosis and mononuclear cell infiltration these paraffin sections were assessed in multiple images taken at 400x magnification on a Zeiss Axioscope A1 microscope (Zeiss, North Ryde, Australia). Image Pro Plus 6.2 software (Media Cybernetics, Rockville, MA) was used to estimate the area of myocytes necrosis and mononuclear cell infiltration, which were expressed as pixels per high power field (HPF).

Immunohistology was performed on 5μM sections of frozen heart allografts cut on a cryostat. Sections were air dried after fixation with acetone for 10min, then stained with a two-step indirect immunoperoxidase technique, as previously described (5, 48). The primary mAb used were W3/25 to CD4, MRCOx8 to CD8 (BD), FJK-16 to Foxp3 and ED1 to CD68 on macrophages (Abcam, Cambridge,cUK), as described (49). The secondary antibodies were HRP labelled anti-mouse Ig (Dako A/s, Glostrup, Denmark). Positive staining was assessed in multiple images taken at 400 X magnification on a Zeiss Axioscope A1 microscope. Image Pro Plus 6.2 software was used to estimate the area of positive staining and was expressed as pixels per high power field (HPF).

Immunostaining of Lymphocytes

FITC labeled anti-rat mAb used were G4.18 (CD3), W3/25 (CD4), MRCOx8 (CD8α), MRCOx39 (CD25, IL-2R alpha chain), MRCOx33 (CD45RA)(BD) and FJK-165 (anti-mouse/rat Foxp3) (eBioscience, San Diego, CA). Staining and analysis of lymphoid cells using a FACScan (BD, San Jose, CA) was as described (19, 50, 51).

Cell Preparation and Subset Separation

Single cell suspensions from spleen and lymph node were prepared as described (50, 52) and RBCs were lysed with a buffer of 0.83% NH_4Cl, 0.1%$KHCO_3$ and 10mM EDTA at pH 7.2. Cells were re-suspended in PBS/0.4% BSA (MultiGel, Biosciences, Castle Hill, NSW, Australia). Spleen and lymph node cells from three or more animals were pooled to provide sufficient $CD4^+CD25^+$ cells for cultures.

An indirect panning technique was used to deplete $CD8^+$T and B cells, as described (14, 50). Briefly, cells were incubated for 30 minutes at 4°C with optimized concentrations of MRCOx8 (an anti-rat CD8α mAb) and MRCOx33 (a rat CD45RA mAb that binds B cells and other cells but not T cells). All mAb were purchased from ThermoFischer. Cells were washed with PBS/

0.4%BSA, re-suspended at $2x10^7$ cells/ml and incubated for an hour on Petri dishes (Greiner Bio-one, Kremsmuenster, Austria) coated with both rabbit anti-mouse Ig and rabbit anti-rat Ig (Dako). The unbound $CD4^+$ cells were collected and incubated at 4°C for 20 min with PE conjugated MRCOx39 (BD) (an anti-rat CD25 mAb), then washed twice before $8\mu l/10_6$ cells were incubated for 15 min at 4°C with of anti-PE microbeads (Miltenyi). Enriched $CD4^+CD25^+$ cells were then eluted through a LS MACS column (Miltenyi) and were re-suspended in RPMI 1640 media with 20% Lewis rat serum for culture. Cell subsets were subjected to immunostaining with mAb. Enriched cells were 97-99% $CD4^+$ and 80-95% $CD25^+$. 60-80% of these $CD4^+CD25^+$T cells were $Foxp3^+$.

For RT-PCR and cell culture in MLC, $CD4^+CD25^+$ T cells were re-suspended in PBS/0.4%BSA.

Assays of Proliferation of $CD4^+CD25^+$T Cells *in MLC*

Stimulator cells were prepared from irradiated (25 gray) thymus cells, as described[19]. In each experiment parallel cultures with self (F344), specific donor (Lewis), third party (PVG) stimulator cells or no stimulator cells were performed. Cell culture medium was RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 100 ng/ml penicillin, 100 U/ml streptomycin (Glaxo, Boronia, Victoria, Australia), 2 mM L-glutamine, $5x10^{-5}$M 2-mercaptoethanol (Sigma), and 20% Lewis rat serum. 20% Lewis rat serum produces low background stimulation in autologous controls (19). Cultures with 5-6 replicates for each experimental group were set up in U-bottom micro-titer plates (Linbro, Flow Labs, VA) containing 2×10^4 stimulators cells and 1×10^5 $CD4^+CD25^+$ cells/well in a total volume of 200 µl. To assess the effects of rIL-5 on proliferation of these cells, 200 U/ml of rIL-5 was added to some cultures, as described (30, 32). Where stated $CD4^+CD25^+$T cells from naïve animals were cultured with rIL-4 (200 units/ml) as described (29).

Cells were cultured at 37°C in humidified air containing 5% CO_2 for 4 days, the peak of $CD4^+CD25^+$T cell proliferation (26). 0.5µCi ^{3}H-thymidine (TRK-120, Amersham, Arlington Heights, IL) was added 16hr prior to harvesting with a Tomtec Cell Harvester (Flow Lab, Ayrshire, Scotland). Proliferation was assayed by adding liquid scintillation fluid before counting on a beta counter (1450 Microbeta Plus, Beckman Instruments, Palo Alto, CA). Each experiment has 5-6 replicates and results were expressed as cpm and presented as mean +/- standard deviation (SD). Counts of <400/min were considered within the range of background

The effect of rIL-5 on $CD4^+CD25^+$T cells proliferation in culture was calculated as a Stimulation Index using the formula: proliferation of cells with rIL-5 to a defined antigen divided by proliferation to the same defined antigen without rIL-5.

RT-PCR of Cytokines and Cytokine Receptors

mRNA extraction from cells and reverse transcription to DNA were as described (21). Primers for rat *Foxp3, Gata3, Tbet, Il2, Il4, Il5, Ifng, Ifngr, Il5ra, Il12rb2 and Gapdh* were as previously reported (28–30, 53). The primers for *Irf4* were F-TGTCCT CCGTGAGCTGTCT; R- CCTGGATCGGCTCCTCTATG, as described (49). **The panel of molecules examined were selected for their relevance to Treg activation**. Real-time PCR was performed as described (54) with a Rotorgene (Corbett Research) and SYBR Green I detection. Sensimix Taq polymerase (BioLine) was used according to manufacturer's instructions. Copy numbers of each gene was derived from a known standard curve performed in parallel and normalized against *Gapdh* expression.

Statistics

Parametric data were expressed as mean ± standard deviation. Results from repeat experiments were pooled, with replicates of ≥ 3 in each experiment. Means were compared using t test with GraphPad Prism (Graphpad Software Inc, La Jolla, CA). Statistical significance was set at $p< 0.05$.

RESULTS

RT-PCR of mRNA From Naïve *$CD4^+CD25^+$* Cells After Culture in MLC With Alloantigen and Type 2 Cytokines

To establish the changes in alloantigen activated Treg during Type 2- responses, we examined cytokines, cytokine receptors and transcription factors that are induced after naïve/resting $CD4^+CD25^+$T cells are cultured first with alloantigen and rIL-4 and later in a second culture with specific alloantigen and rIL-5. The experimental protocol is illustrated in **Figure 2A**. The hypothesis was that rIL-4 and alloantigen would activate naïve/resting $CD4^+CD25^+$Treg to Ts2 cells expressing IL-5Rα that would proliferate when stimulated by specific-alloantigen and rIL-5 (29, 30, 32) and develop into Th2-like Treg.

$CD4^+CD25^+$cells from naïve DA rats were cultured for 4 days with fully allogeneic PVG stimulator cells and 200 units of rIL-4 as described (29) to induce Ts2 cells. These Ts2 cells were washed and re-cultured with 200 units of rIL-5 and the same alloantigen to induce Th2-like Treg. Combined results from three separate experiments of RT-PCR of mRNA of these cells are shown in **Figure 2B**. The Th2-like Treg had increased expression of mRNA for the transcription factors *Foxp3, Irf4*, and *Gata-3*, but had no induction of *tbet. il5* but not *il4, il2* or *ifng* was induced in these cells. Compared to starting $CD4^+CD25^+$ cells from naïve rats where naïve/resting tTreg ($CD4^+CD25^+Foxp3^+$Treg) forms a major part, *Il5ra* expression was increased in Ts2 cells, but this increase was not sustained in Th2-like Treg. We used expression of *Irf4* and *Il5* as markers of Th2-like Treg induction.

Effect of rIL-5 Treatment on Lewis Heart Graft Rejection in F344 Rats

Our hypothesis is that during a rejection response, some Th2 cells will be activated to produce IL-4 that with donor antigen would activate naïve/resting $CD4^+CD25^+Foxp3^+$Treg to Ts2 Treg as proposed in **Figure 1**. Lewis heterotopic cardiac allografts into F344 rats are slow to reject as there is only a

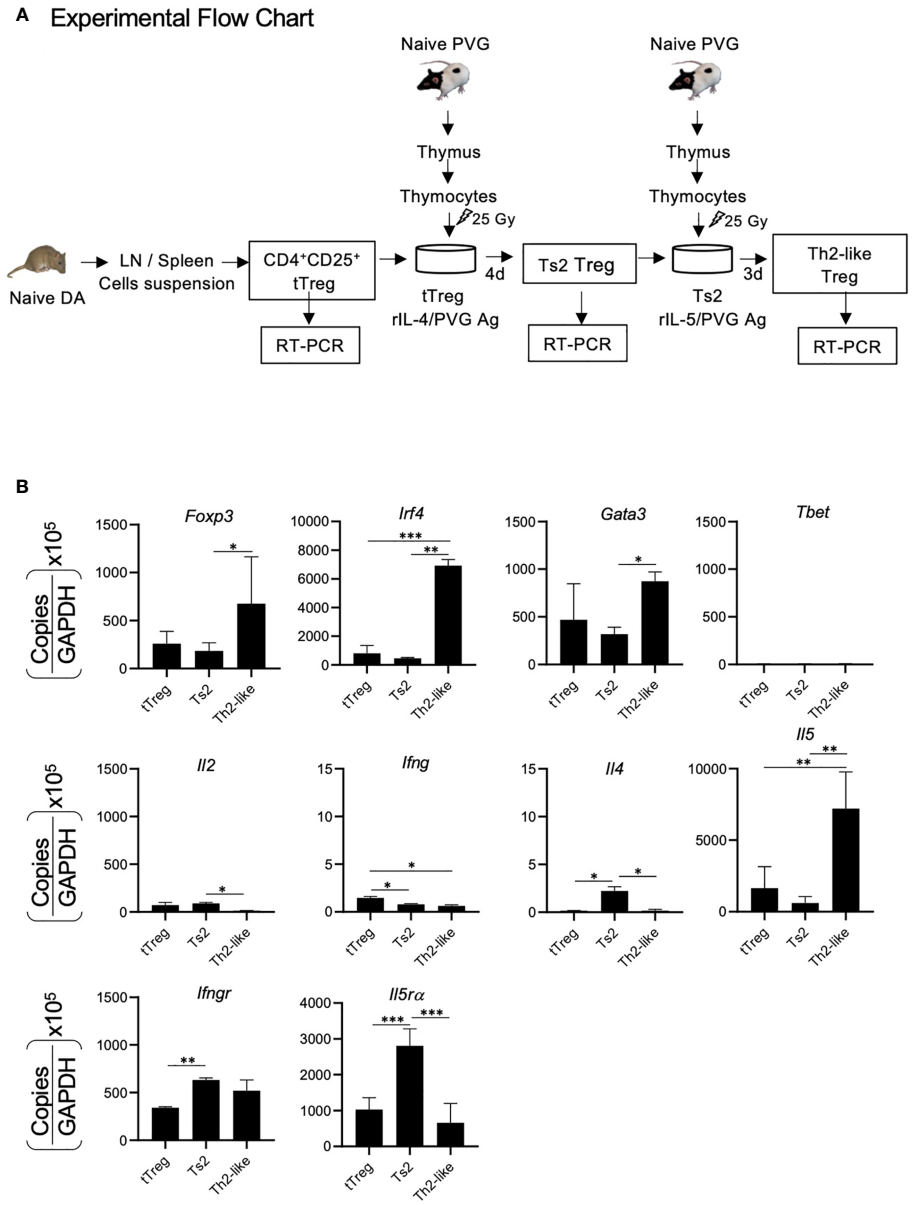

FIGURE 2 | RT-PCR of mRNA from $CD4^+CD25^+$T cells demonstrating changes in naïve $CD4^+CD25^+$T cells cultured with alloantigen and Type-2 cytokines. **(A)** Experimental Flow Chart. $CD4^+CD25^+$T cells from naïve DA rats were enriched and cultured with fully allogeneic PVG thymic stimulator cells and rIL-4 for 4 days to induce Ts2 cells that express Il-5Rα. These Ts2 cells were re-cultured for 3 days with same alloantigen and rIL-5 to induce the Th2-like Treg. RT-PCR was performed on mRNA from fresh naïve $CD4^+CD25^+$Treg and cultured activated Ts2 and Th2-like Treg to examine for transcriptions factors *Foxp3, T-bet, Gata-3, Irf4;* cytokines *Il2, Il4, Il5, Ifng* and cytokine receptors *Il5ra, Ifngr*. **(B)** Results of RT-PCR of tTreg, Ts2 and Th2 like Treg. Data shown is a combination of results from three separate experiments. Data expressed as copies for relevant molecule divided by copies of Gapdh, multiplied by $10^{5.}$ *p < 0.05, **p < 0.01, ***p < 0.001. The Th2-like Treg had greater expression of *Foxp3, Irf4* and *Il5* than fresh naïve $CD4^+CD25^+$T cells and Ts2 cells. They had more *Gata-3* than Ts2 cells. *Il5ra* was induced in Ts2 cells but was not sustained in Th2-like Treg. *T-bet, Ifng* and *Il4* were low in all samples and there was minimal *Il2* (<100 copies). Expression of *Irf4* and *Il5* were used as markers of Th2 like Treg. The changes associated with Th1-like Treg have been described (28).

single class I MHC and multiple minor incompatibilities with no class II MHC incompatibility (27, 28). The model is delayed acute rejection with T cell activation and infiltration. The experimental plan is shown in **Figures 3A** and **4A**. Graft function was scored using a semi-quantitative scale described in methods and mean heart graft function score are presented on y-axis (**Figures 3B**, **4B**).

Rejection in sham treated hosts (n=8) caused a decline in graft function after day 10, with complete rejection by day 31 (**Figure 3B**). No rats in this sham treated control group

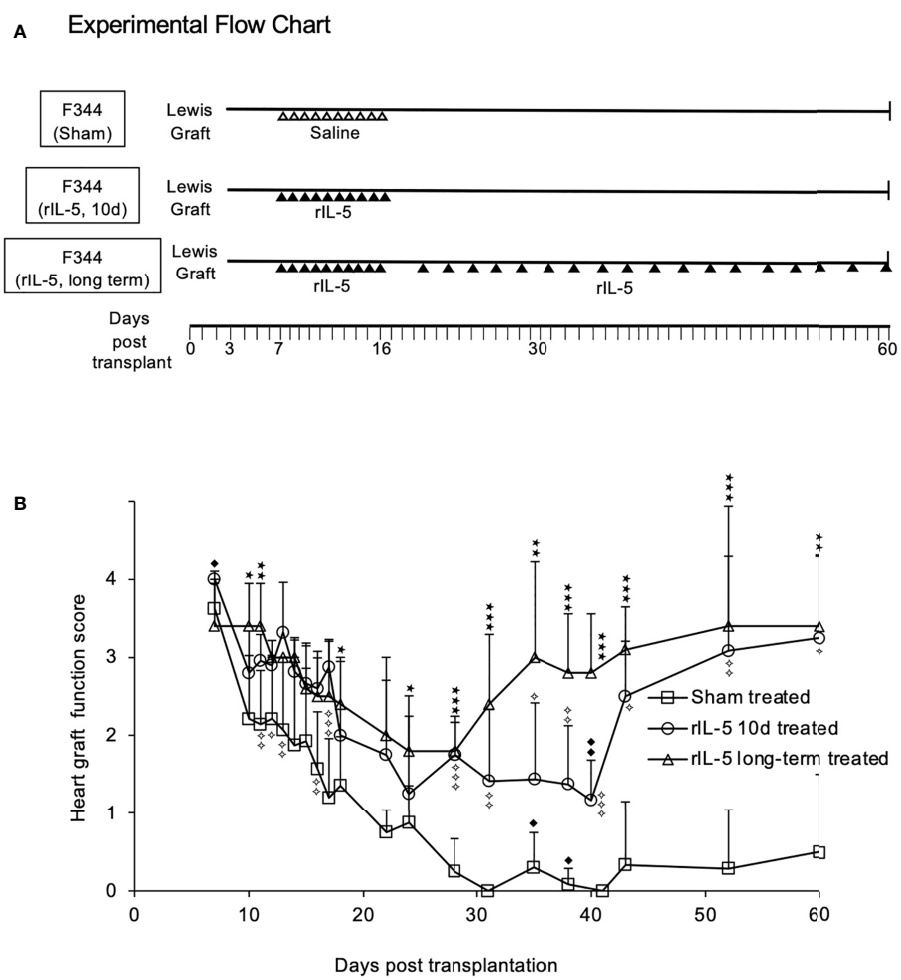

FIGURE 3 | IL-5 treatment prevents rejection of Lewis heart grafts in F344 hosts. **(A)** Experimental Flow Chart. Groups of F344 rats with heterotopic Lewis heart grafts were studied. Sham treated rats received saline (ipi) daily from day 7-16 post-transplantation. One treatment group (short-term rIL-5) received 5000 units/day of rIL-5 (ipi) from day 7 to 16 post-transplant. The second treatment group (long term IL-5) received rIL-5 therapy 5000 units/day from day 7-16 and beyond 16 days had three ipi of 5000 units rIL-5 per week until 60 days post transplantation. **(B)** Monitoring of F344 rats with Lewis heterotopic cardiac allografts. Severity of rejection was assessed by palpation and scored on a semi-quantitative scale, described in methods. Severity of rejection scores in each group was expressed as mean ± standard deviation. Sham treated (□) received daily injections of 0.5ml of normal saline ipi starting at day 7 through to day 16 (n=5). All grafts had severe rejection by day 21. After 28 days, the mean score was <0.5 and there was no recovery of graft function. Short term rIL-5 treatment for 10d (○). 5000 units of rat rIL-5 was given daily ipi in 0.5ml from day 7 through to day 16 (n= 12), as described (22). Rejection was significantly less than sham treated on day 16 ($p<0.01$) and at day 17 ($p<0.001$). From day 28, graft function improved and was significantly greater than sham treated controls until day 60 ($p<0.05$). Significance compared to sham treated controls; ✧ $p<0.05$, ✧✧ $P<0.01$; ✧✧✧ $p<0.001$. Long-term rIL-5 treated (△). 5000 units of rat rIL-5 in 0.5ml was given ipi in 0.5ml daily from day 7 through to day 16, then 3 times a week until day 60 (n= 5). Rejection in this group was significantly less ($p<0.01$) than sham treated group from days 19 until day 63. Rejection was significantly less than the group treated with rIL-5 for 10 days from day 32 until day 50 ($p<0.05$). The long-term rIL-5 treated group had less rejection than the short-term rIL-5 treated group at day 34 and day 38 ($p<0.05$) and at day 40 ($p<0.01$). By day 43, both rIL-5 treated groups had a mean graft score of *3*. Significance compared to sham treated; $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$. Significance compared to 10 day rIL-5 treated ◆ $p<0.05$, ◆◆ $p<0.01$.

recovered to have significant function, with graft function scores of 0.5 or 0.

Short-term rIL-5 treatment (n=12) was 5000 units ipi daily for 10 days between 7 and 16 days post-transplantation (**Figure 3A**). In the long-term treatment group (n=5), rIL-5 therapy was continued (ipi) three times a week immediately following the daily rIL-5 from 7 to 16 days post-transplant (**Figure 3A**). In both rIL-5 treated groups, graft function scores were higher than in sham treated rats at all time points beyond day 10 post-transplant ($p<0.01$)(**Figure 3B**). rIL-5 treatment preserved graft function, with all grafts scoring ≥2 until cessation of rIL-5 treatment at day 16. The graft function score was significantly higher than sham treated group, $p<0.01$ at day 16 and $p<0.001$ at day 17 (**Figure 3B**).

Heart graft function in both rIL-5 treatment groups stabilized around 20 days post-transplant then improved. The group that

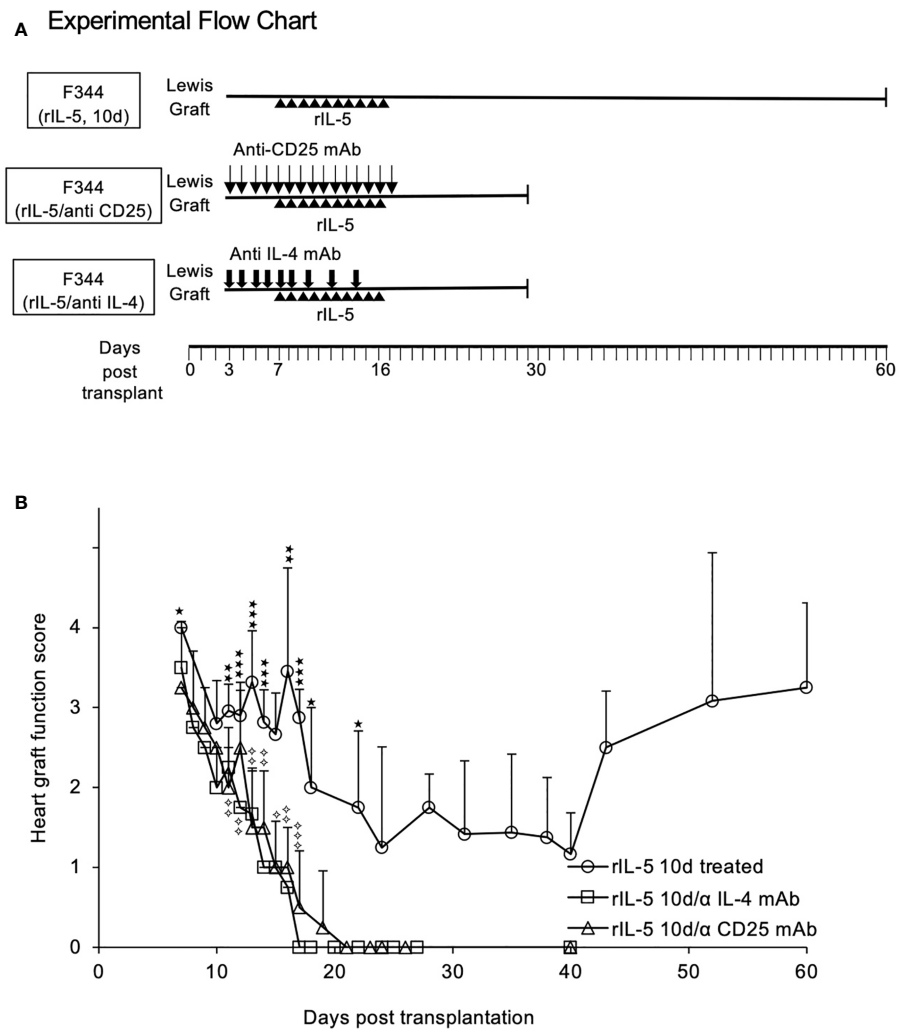

FIGURE 4 | Blocking IL-4 or depleting $CD25^+$ cells prevents rIL-5 treatment inhibiting rejection of Lewis heterotopic heart allografts in F344 recipients. **(A)** Experimental Flow Chart. All groups received 5000 units of rat rIL-5 daily (ipi) in 0.5ml. One group was treated with anti-CD25 mAb (NDS61) (n=4) and another with MRC OX81 (n=4), as described in methods. A control group received rIL-5 treatment from day 7-16 post transplantation, as in **Figure 3**, with no blocking mAb (○). Rejection scores are expressed as mean ± standard deviation. **(B)** Graft survival anti-CD25 mAb treated. Anti-CD25 treated (△) rats had more severe graft rejection than rIL-5 treated alone with significant differences from day 15 onwards to day 40 post-transplant ($p<0.05$). All grafts had fully rejected by day 19, and none recovered. Significance compared to rIL-5 treated controls; ✧ $p<0.05$, ✧✧ $p<0.01$; ✧✧✧ $p < 0.001$. Graft Survival anti-IL-4 treated. Animals treated with anti-IL-4 mAb (□) rejected their transplants more rapidly than rats treated with rIL-5 alone, with significant differences from day 9 ($p<0.05$) and on all subsequent days ($p<0.01$). All grafts were fully rejected by day 17 and none recovered. Significance compared to rIL-5 treated controls; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Taken together, these studies showed host $CD25^+$T cells, presumably naïve Treg, were required as was host IL-4 to induce a state where rIL-5 therapy could delay rejection and promote long-term allograft survival.

received long-term treatment with rIL-5 therapy had more rapid improvement in graft function, with scores significantly higher than sham treated controls at all time points beyond day 22 ($p<0.05$). Compared to 10-day treatment group, the long-term rIL-5 treated group had higher graft function scores on day 35 ($p=0.05$) and 40 ($p<0.01$) (**Figure 3B**). By day 43, both rIL-5 treated groups had a mean graft function score of 3. At the end of monitoring on day 60, 3 of 5 long-term rIL-5 treated rats had an excellent graft function score of *4* and another rat had a score of *3* (**Figure 3B**). This level of heart graft function is consistent with operational transplant tolerance and similar to long-term syngeneic heart graft function in this rat allograft model, as observed in previous studies.

Depletion of $CD25^+$ Cells Prevented rIL-5 Treatment Slowing Rejection

The rationale for rIL-5 treatment was to expand $CD4^+CD25^+$ $Foxp3^+$Treg that had been activated by IL-4 produced in the early rejection response. NDS61, a mAb to rat CD25, depletes $CD4^+CD25^+$T cells in rats (47) and prevents rIL-5

treatment inhibiting autoimmune responses (30, 32). To demonstrate a role for $CD25^+$ cells, we depleted these cells by ipi of NDS61 daily from 3 to 17 days post-transplantation, as illustrated in **Figure 4A**. Hosts depleted of $CD25^+$T cells and treated with rIL-5 rejected their allografts faster, with all allografts fully rejected at day 19 (n=4) (**Figure 4B**). No graft function was detected in any animal treated with NDS61 and rIL-5 after day 20 and there was no recovery in graft function in the next 10 days. Graft rejection was more severe in anti-CD25mAb and rIL-5 treated rats (**Figure 4B**) than in sham treated from day 14 to 19 ($p<0.05$) (**Figure 3B**). This suggests $CD25^+$cells are activated during rejection and slow the progress of rejection.

Blocking IL-4 Inhibited the Effects of rIL-5 Treatment on Preventing Rejection

MRCOx81, a mAb that blocks IL-4 (40, 41), was administered daily from day 3-8 then on days 10,12,14 post-transplantation, as illustrated in **Figure 4A**. Anti-IL-4 mAb treatment also led to accelerated rejection and abolished the effect of rIL-5 treatment on allograft survival (n=4) (**Figure 4B**). All rats totally rejected their heart grafts by day 17 and there was no recovery in graft function over the next 10 days. All MRCOx81 treated rats rejected faster than sham treated controls (**Figure 3B** and **Figure 4B**). MRCOx81 and rIL-5 treated group had significantly lower graft function scores (**Figure 4B**) compared to those from rats treated with rIL-5 alone, on all monitoring days from day 11 ($p<0.01$)(**Figure 4B**).

Histology of Heart Grafts in rIL-5 Treated Hosts: rIL-5 Treatment Reduced Mononuclear Cell Infiltration and Damage to Myocytes

The experimental protocol for obtaining Lewis heart graft tissue from F344 rats for histology is illustrated in **Figure 5A**. Heart grafts from rIL-5 treated rats taken at day 16 post-transplant had good cardiac myocyte preservation with scattered mononuclear cells infiltration (**Figure 5B**). In contrast, grafts from sham treated rats had wide-spread myocyte necrosis and large infiltrates of mononuclear cells. Additional examples are in **Supplementary Figure 1**. Donor Lewis hearts in F344 recipients treated with rIL-5 and either anti-CD25 or anti-IL-4 mAb had massive areas of myocyte necrosis with dense infiltrates of mononuclear cells, however these heart grafts were taken at the end of the experiment at day 30 not at day 17 post-transplant (**Figure 5B**).

Image analysis of donor hearts showed the pixels occupied by necrotic myocytes was less in rIL-5 treated 139,475 ± 35,078 than in sham treated controls 474,969 ± 154,423 ($p=0.00011$); anti-IL-4 mAb and rIL-5 treated 436,217 ± 138,148 and anti-CD25 mAb plus rIL-5 treated 536,889 ± 272,577 (**Figure 5C**). The area of mononuclear cells measured by pixels was less in rIL-5 treated 193,883 ± 108,701 than in sham treated controls, 311,4112 ± 124,968 ($p=0.03$), anti-IL-4 mAb and rIL-5 treated 269,521 ± 35,636 and anti-CD25 mAb plus rIL-5 treated 249,281 ± 102,820 (**Figure 5C**). The mAb treated animals grafts were collected two weeks longer post-transplant and had established rejection, thus this data is not directly comparable.

Characterization of the mononuclear cell infiltrate in heart grafts using immunostaining with mAb, (**Figure 5D**), showed that compared to grafts from sham treated hosts that were rejected, rIL-5 treated grafts had significantly fewer $CD8^+$ cells ($p=0.02$), $CD4^+$ cells ($p<0.05$) and $Foxp3^+$ cells ($p= 0.05$). 44% of $CD4^+$ cells in IL-5 treated expressed Foxp3, whereas 41.9% expressed Foxp3 in rejected grafts. Representative sections are shown in **Supplementary Figure 2**. There was no difference in the infiltrate of $ED1^+$ macrophages between sham treated and rIL-5 treated.

Thus, rIL-5 treatment preserved the heart graft from injury and markedly reduced the mononuclear cell infiltrate compared to grafts from sham-treated rats. The benefits of rIL-5 treatment were abolished by treatment with anti-CD25 mAb or anti-IL-4 mAb (**Figures 5A–D**).

Effect of Treatment With rIL-5 for 10 Days on $CD4^+CD25^+$T Cells in Peripheral Lymphoid Organs of Hosts

The source of lymphocytes for these studies is illustrated in **Figure 6A**. We examined $CD4^+CD25^+$ cells from graft bearing hosts to examine if IL-5 administration resulted in *in vivo* activation of Ts2 cells and/or Th2-like Treg as assessed by *in vitro* proliferation with rIL-5 (**Figures 6B–D**) and RT-PCR of key markers (**Figures 7A, B**).

Spleen and lymph node cells from rats treated with rIL-5 for 10 days were examined either at the end of rIL-5 treatment on day 16 (**Figure 6B**), or on day 66 (**Figure 6C**) post-transplantation. Cells from sham treated F344 rats with Lewis heart graft were assessed at 56 days post-transplantation (**Figure 6D**). The proportion of $CD4^+CD25^+$cells in rIL-5 treated rats was 6.3-7.8% (**Figures 6B, C**) compared to 4% in sham-treated controls (**Figure 6D**). $Foxp3^+$ cells were 2.8% - 4% in rIL-5 treated rats and 6.6% in sham-treated controls (data not shown).

The enriched $CD4^+CD25^+$ cells in all three groups were 80-88% $CD4^+CD25^+$cells (**Figures 6B–D** left panel), 94-99% $CD3^+$, <2.4% $CD8^+$, and 61-70% $Foxp3^+$ cells (data not shown). This is within the standard enrichment of murine Treg using CD25. Thus, 30-40% of cells in the enriched $CD4^+CD25^+$T cells were $Foxp3^-$ and not necessarily T regulatory cells. They may include activated effector $CD4^+$T cells.

Enriched $CD4^+CD25^+$ cells were tested for proliferation in MLC (**Figures 6B–D**, middle and right panel) and their mRNA tested by RT-PCR (**Figures 7A, B**). Enriched $CD4^+CD25^+$T cells from rIL-5 treated hosts taken 16 days post-transplant (**Figure 6B**, middle panel), in absence of rIL-5 in culture, had a greater response to Lewis, than to self (F344) or third party. Such proliferation to graft alloantigen suggested increased numbers of cells activated by graft alloantigens. rIL-5 in cultures partially enhanced responses to specific donor Lewis, but this was not significant, as seen in the Stimulation Index

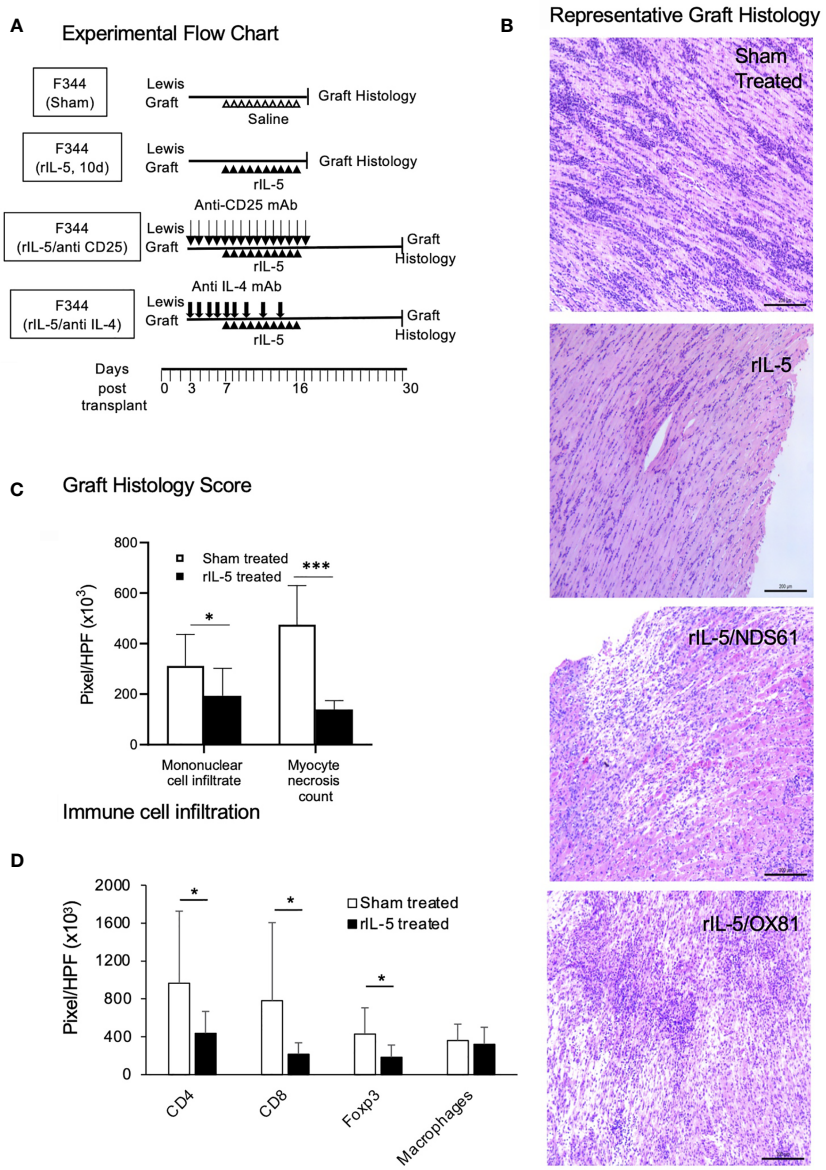

FIGURE 5 | rIL-5 treatment reduces myocyte necrosis and mononuclear cell infiltrates in Lewis heart grafts transplanted to F344 hosts. **(A)** Experimental Flow Chart. Representative animals from sham treatment and 10 day rIL-5 treatment (**Figure 3**) groups were sacrificed for study of histology, myocyte damage, and mononuclear cell infiltrates in their cardiac allogafts. Some animals from anti-CD25 and anti-IL-4 mAb therapy together with rIL-5 treatment groups (**Figure 4**) were sacrificed for histological studies. **(B)** Photomicrographs of H&E sections of Lewis cardiac allografts from F344 hosts, Samples were taken at days 17-19 post-transplant shortly after daily rIL-5 treatment had stopped. Images taken by a Leica DFC 450C camera with 400X magnification on a Leica DM 2000 LED microscope. Heart grafts from sham-treated hosts had large areas of mononuclear cell infiltration and scattered infiltrate between myocytes (Top panel). There were wide areas of myocyte necrosis. Heart grafts from animals treated with rIL-5 had minimal mononuclear infiltration between myocytes and minimal myocyte necrosis (second panel). Grafts from hosts treated with rIL-5 that also received NDS61 an anti-CD25 mAb to deplete Treg (third panel) and MRCOX81 mAb to block IL-4 (bottom panel) had large areas of myocyte necrosis and cell infiltration. **(C)** Areas of mononuclear cell infiltrate and myocyte necrosis was assessed as pixels per high power field (HPF) in multiple images taken at 400 X magnification on a Zeiss Axioscope A1 microscope (Zeiss, North Ryde, Australia) using Image Pro Plus 6.2 software (Media Cybernetics, Rockville, MA). Data expressed as mean ± SD, *$p < 0.05$, ***$p < 0.001$. Area of mononuclear cell infiltration measured in pixels was significantly less in grafts in rIL-5 treated rats than those from sham treated rats; 193,883 ± 108,701 *vs* 311,4112 ± 124,968 ($p = 0.03$) (**Figure 3C**). Area of myocytes necrosis measured in pixels was significantly lower ($p<0.00011$) in heart grafts from rIL-5 treated rats than in hearts from sham treated controls (**Figure 3C**). **(D)** Immunostaining for mononuclear cells in heart grafts from F344 rats showing comparison of cell infiltrate in grafts from rIL-5 treated animals to those from sham treated. Grafts from rIL-5 treated animals had significantly reduced area of $CD4^+$ cells ($p<0.05$), $CD8^+$ cells ($p<0.05$), and $Foxp3^+$ cells ($p<0.05$) compared to those from sham treated animals. The ratio of $Foxp3^+$ cells in $CD4^+$ cells was similar in rIL-5 treated and sham-treated rejection controls. There was no difference in infiltration of $ED1^+$ macrophages. Photomicrographs of these stained sections are in **Supplementary Figure 2**.

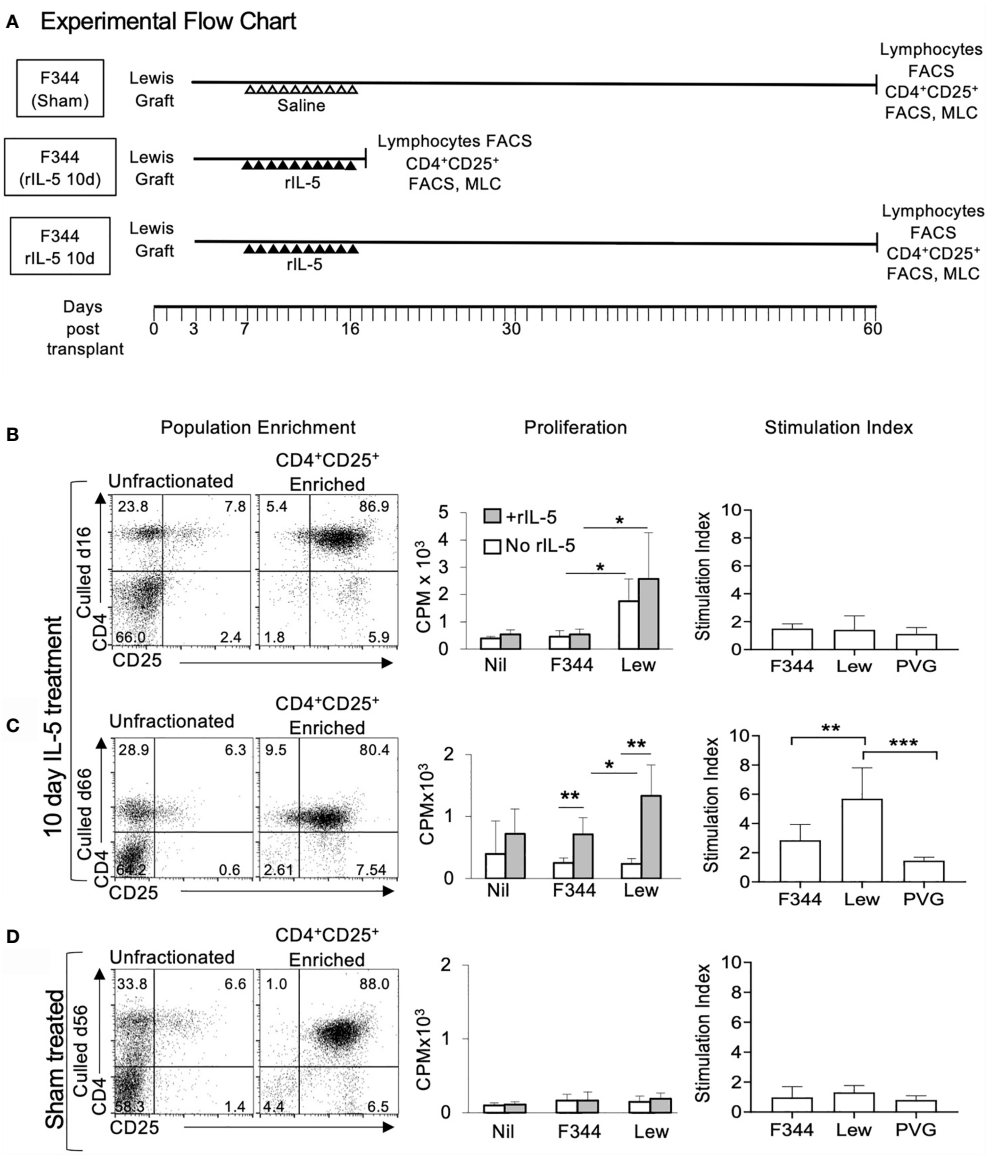

FIGURE 6 | Flow cytometry profiles and proliferation of $CD4^+CD25^+$ T cells from spleen and lymph nodes from F344 recipients of Lewis cardiac allografts. **(A)** Experimental flow chart with animal treatment and collection time of peripheral lymphoid tissues for FACS and MLC. All cell donors were F344 grafted with a Lewis heterotopic heart transplant. Cells from rIL-5 treated were taken at 16 days post transplantation at the end of 10 days rIL-5 treatment, or at 66 days post-transplant. Sham treated recipients' cells were taken at 56 days post-transplant. **(B–D)** Enriched $CD4^+CD25^+$T cells (left column) from lymph nodes and spleens from Lewis allograft bearing F344 recipients were examined for their capacity to proliferate in MLC in response to no stimulator cells (Nil), or stimulator cells from self (F344), specific donor (Lewis) or third party PVG. The understanding of the current findings is dependent upon our previous findings. First, naïve $CD4^+CD25^+$T cells, in the absence of rIL-2 or IL-4 have a very small response to alloantigen, and none to self. $CD4^+CD25^+$T cells from tolerant hosts do not respond to the tolerated donor strain but they do respond to third party. The proliferation of $CD4^+CD25^+$T cells from tolerant hosts to specific donor, but not to self or third party is enhanced by addition of cytokines such as rIL-5. Effect of rIL-5 on proliferation of $CD4^+CD25^+$T cells to self and specific donor is shown in middle column. Proliferation to third party PVG, which is fully allogeneic, is much larger to self and Lewis (data not shown). Stimulation indices were calculated as proliferation with rIL-5 in culture divided by the proliferation to the same donor stimulator cells with no rIL-5 (n=-6). **(B)** Cells from hosts treated with rIL-5 taken at day 16 post-transplant. $CD4^+CD25^+$T cells represented 7.8% of unfractionated lymphocytes and 87% of enriched cells (left panel). The enriched $CD4^+CD25^+$ T cells proliferated to specific donor, but not to self (middle panel). This proliferation was slightly enhanced by adding rIL-5 to cultures, but not significantly when assessed as Stimulation Index (right panel). **(C)** Cells from hosts treated with rIL-5 for 10 days and culled 66 days post-transplant, had 6.3% $CD4^+CD25^+$ cells (left panel). $CD4^+CD25^+$T cells did not respond to self or specific donor (middle panel). The proliferation to specific donor was enhanced significantly by adding rIL-5 to the culture (middle panel) as illustrated by Stimulation Index (right panel). The response to self or third-party was not enhanced by rIL-5 (right panel). This is consistent with our hypothesis that alloantigen specific $CD4^+CD25^+$T cells become dependent upon IL-5 for expansion. **(D)** Cells from hosts given sham treatment taken at 56 days post-transplantation had similar proportions of $CD4^+CD25^+$ cells (6.6%) to animals treated with rIL-5. However, these cells did not respond to specific donor alone, and rIL-5 did not enhance proliferation (middle and right panel), indicating absence of alloantigen-specific Treg that depend upon IL-5. *$p < 0.5$, **$p < 0.01$, ***$p < 0.05$.

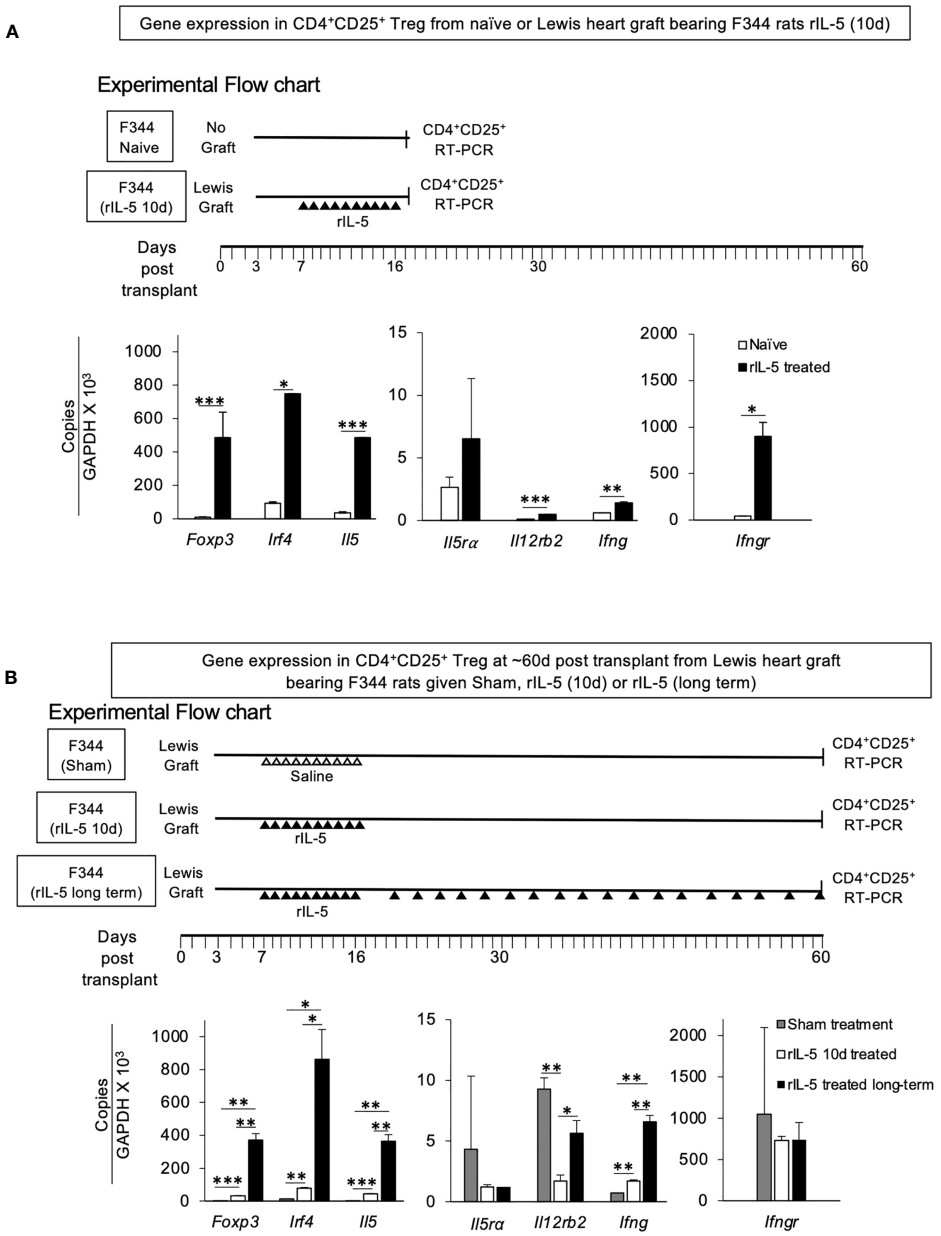

FIGURE 7 | RT-PCR assays of mRNA for transcription factors, cytokines and cytokine receptors in $CD4^+CD25^+$T cells from F344 rats. **(A)** Comparison of RT-PCR of mRNA of $CD4^+CD25^+$T cells from naïve F344 to those from F344 rats bearing Lewis heart graft treated with rIL-5 for 10 days. $CD4^+CD25^+$ cells were enriched from lymph node and spleens of F344 rats as described in methods and subjected to mRNA extraction. mRNA prepared at 16 days post transplantation from F344 rats bearing Lewis heart grafts and treated with rIL-5 for 10 days was compared to that from naive F344 rats that had no transplant and no treatment (Experimental Flow Chart). mRNA was subjected to cDNA extraction followed by RT-PCR of transcription factors, cytokines and cytokine receptors associated with activation of naive $CD4^+CD25^+$ T cells by alloantigen and either IL-2 (*Ifngr, Il12rb2)* or IL-4 (*Irf4* and *Il5ra*). $CD4^+CD25^+$T cells from rIL-5 treated graft bearing hosts had more *Foxp3* consistent with activation of Treg, and more *Il5*, and *Irf4*, consistent with induction of Th2-like Treg. There was also induction of *Ifngr, Ifng* and *Il12rb2* consistent with induction of Treg by Type-1 cytokines (28, 29). *p < 0.05, **p < 0.01, ***p < 0.001. **(B)** Comparison of RT-PCR of mRNA from $CD4^+CD25^+$ cells from F344 hosts with Lewis heart grafts at >56 days. $CD4^+CD25^+$T cells from spleen and lymph nodes of F344 rats treated with rIL-5 for short term (10 days) or long-term were compared to those from sham treated hosts (Experimental Flow Chart). $CD4^+CD25^+$T cells in long-term rIL-5 treated animals were not increased and was ~6.4% (data not shown), similar to those from short-term rIL-5 treated and sham- treated rats (**Figure 6**). $CD4^+CD25^+$T cells from long-term treatment group had greater expression of *Foxp3, Irf4* and *Il5* than cells from recipients where rIL-5 treatment was given only for 10 days that stopped at 16 days post transplantation. Cells from sham treated rats had less expression of Th2-like Treg markers than those from rats given short term rIL-5 treatment. Thus, continued rIL-5 treatment strongly retained the Th2-like Treg phenotype. *p < 0.05, **p < 0.01, ***p < 0.001.

(**Figure 6B**, right panel). rIL-5 in culture also did not enhance the response to self (F344) or third party (PVG) (**Figure 6B**, middle and right panel).

Enriched $CD4^+CD25^+$ cells (>88%) from F344 rats with Lewis heart grafts, treated for 10 days with rIL-5, at day 66 post-transplant had no proliferation to specific-donor in the absence of rIL-5 (**Figure 6C**, middle panel). This was consistent with our previous observations that Treg from rats with transplant tolerance do not proliferate to specific-donor in the absence of key cytokines (26, 31). rIL-5 in culture enhanced their proliferation to specific-donor Lewis with a Stimulation Index that was signifiantly greater than that to self ($p<0.01$) and third-party PVG ($p<0.001$) (**Figure 6C** right panel). Proliferation to self or to third-party was not enhanced by addition of rIL-5. (**Figure 6C**, right panel).

$CD4^+CD25^+$ cells from sham-treated hosts, alone or with rIL-5 had no proliferation to specific donor, self or third party simulators (**Figure 6D**, middle panel). These animals had rejected their grafts and would not be expected to have activated Treg surviving 56 days post-transplant. Cultures of $CD4^+CD25^+$ cells from rats receiving long-term rIL-5, taken at day 60 post-transplantation, failed due to malfunction of an incubator and could not be repeated due to animal ethics issues.

RT-PCR of mRNA for Transcription Factors, Cytokines and Cytokine Receptors on $CD4^+CD25^+$ Cells From F344 Rats With Lewis Heart Grafts

RT-PCR of mRNA from $CD4^+CD25^+$T cells from peripheral lymphoid tissue of heart grafted animals taken on day 16 at the end of treatment for 10 days with rIL-5 was performed. Controls were $CD4^+CD25^+$ cells from naïve F344 animals that had not been transplanted with a heart graft and had no treatment (**Figure 7A**). $CD4^+CD25^+$T cells from heart graft recipients treated with rIL-5 had significantly greater expression of *foxp3* ($p<0.001$), *Irf4* ($p<0.05$), *Il5* ($p<0.001$), and higher *Il5Ra* (not significant) compared to $CD4^+CD25^+$T cells from naive F344 rats. These findings suggested that rIL-5 treatment activated Th2-like Treg, which expressed *Irf4* and *Il5*. There was also induction of Th1-like Treg markers *Ifngr* ($p<0.05$), *Il12rb2* (p,0.001) and *Ifng* ($p<0.01$), showing Th1-like Treg were also present.

$CD4^+CD25^+$ cells from rIL-5 and sham treated rats were also compared at around 60 days post transplantation (**Figure 7B**). $CD4^+CD25^+$ cells from long-term rIL-5 treated rats on day 60 post-transplantation, expressed more mRNA for *Foxp3* ($p<0.01$), *Irf4* ($p<0.05$) and *Il5* ($p<0.01$) than those from sham-treated rats with heart grafts, at 56 days post-transplantation. Sham treated rats did not receive rIL-5 and had rejected by day 31 post-transplant. The cells from short-term rIL-5 treated group (66 days post-transplantation), also had an increase in *Foxp3* ($p<0.001$), *Irf4* ($p<0.01$) and *il5* ($p<0.001$) compared to sham-treated at day 56 post-transplantation. Expression of Th1-like Treg marker *Ifng* ($p<0.01$) was also increased, but *Ifngr* and *Il12rb2* were not (**Figure 7B**).

DISCUSSION

Treatments to promote transplant tolerance could improve long-term allograft survival in patients. Immunoregulation is a complex process that involves a number of Treg pathways (23, 27, 43). In rodent models transplant tolerance is mediated by $CD4^+CD25^+$T cells (16, 17) that express Foxp3. During immune response to newly transplanted tissues, a variety of T effector cells are activated and can mediate rejection, including Th1 (44), Th2 (44, 45) and Th17 cells (46, 52, 55). Cytokines produced by these activated Th cells promote distinct pathways of activation of $CD8^+$T cells, macrophages, and B cells. This study adds to a growing body of work showing they also activate distinct tTreg pathways (23, 28, 29, 43) to produce different subclasses of Treg, which contribute to tolerance to an allograft (43).

Resting/naïve tTreg are activated during all rejection responses, and can, if the graft is not totally destroyed, dominate rejection and allow recovery of graft function. In the model used, Lewis grafts in F344 recipients, one in ten grafts undergo transient rejection but fully recover and have good function long-term. This process is usually dominated by Type-1 activated Treg. In this study, rIL-5 treatment delayed rejection and allowed this natural regulatory process to dominate, permitting all grafts in rIL-5 treated host to survive without any immunosuppression. Thus, Type-2 activated Treg augmented the Type-1 activated Treg to inhibit the rejection response and promote tolerance to the allograft. In the short term rIL-5 treated the cessation of rIL-5 resulted in more rejection, however over time the grafts recovered and gained function similar to long term rIL-5 treated. We attributed this rejection to loss of IL-5 to promote Ts2 cells. The later recovery occurs if rejection is not total and is seen in a small proportion of F344 rats with Lewis heart grafts. We attribute this to induction of activated Treg by the grumbling rejection response.

Apart from delaying complete allograft rejection, less myocyte necrosis and mononuclear cells infiltration was identified in grafts in rIL-5 treated hosts. There was significantly fewer $CD4^+$, $CD8^+$, $Foxp3^+$ infiltrating T cells in grafts of rIL-5 treated rats but macrophage infiltration was not reduced.

The best-defined pathway of activation of naïve/resting $CD4^+CD25^+Foxp3^+$ tTreg involves Type-1 cytokines. This activation is a two-step process. In the first step, IL-2 activates naïve/resting tTreg, and in the presence of alloantigen generates donor-specific activated Treg (28, 29, 50, 51) that express receptors for Type-1 cytokines, including IL-12 and IFN-γ (28, 29, 51). We identified these Ts1 cells by using Type I cytokines to activate $CD4^+CD25^+$cells from a naïve host *in vitro* (28, 29, 51). In the second step of activation the phenotype of Ts1 cells can be further modified by stimulation to specific donor alloantigen and IL-12 (28) in the absence of IL-2. This second step induces Th1-like Treg to express both Foxp3 and *Tbet*, also to produce *Ifng* but not *Il2*. These Th1-like Treg are much more potent at suppressing rejection than tTreg or Ts1 cells (28). Th1-like Treg markedly delay rejection of fully allogeneic heart grafts (28).

$CD4^+CD25^+$ cells from animals with transplant tolerance, which includes alloantigen specific Treg, do not proliferate to

specific donor alloantigen *in vitro*, but they can proliferate to donor alloantigen if either IFN-γ or IL-12 are present in the culture medium (28, 29). Treatment with rIL-12 in some models can delay rejection of an allograft, and this effect requires IFN-γ (54). IFN-γ has been shown by others to promote expansion of antigen-specific Treg (56, 57). Thus, IL-12 and/or IFN-γ may promote induction of Th1-like Treg and promote tolerance. Both of these Type-1 cytokines have the potential to also promote Th1 responses and rejection, however.

Relevant to this study is the pathway of activation of naïve/resting $CD4^+CD25^+$ cells by antigen and Type-2 cytokines (**Figure 1**) (29, 30, 32, 43). In the first step, tTreg activated by alloantigen and IL-4, in the absence of rIL-2 in the culture, are induced to express mRNA for the receptor of the Type-2 cytokine IL-5, not the receptors for Type-1 cytokines IFN-γ and IL-12 (29). These activated tTreg we named Ts2 cells (29). In a second step, Ts2 cells further proliferate in the presence of IL-5 and specific alloantigen to become Th2-like Treg. Therapy with rIL-5 inhibits acute allograft rejection and induction of Th1 and Th17 responses (34) and promotes Ts2 cells (30, 32). IL-5 promotes survival of transplant tolerance transferring $CD4^+$T cells, which die *ex vivo* without key cytokines, one of which is IL-5 (33). $CD4^+CD25^+$ cells from animals with transplant tolerance do not respond to specific donor alloantigen unless key cytokines such as IL-5 are present (31). In this study, $CD4^+CD25^+$ cells from rIL-5 treated, but not sham treated, hosts had a proliferative response to specific donor that was enhanced by rIL-5 in culture.

In this study, the Type-2 cytokine milieu did not inhibit induction of Ts1 and Th1-like Treg especially early on at day 16. Longer term, at around 60 days post-transplant where rIL-5 therapy was stopped at day 16 post-transplant, molecules associated with Th1-like Treg were also induced in $CD4^+CD25^+Foxp3^+$Treg. Thus, Type 1 and Type 2 activated Treg can be activated in parallel and are not mutually exclusive.

For some time, Th2 responses were thought to promote transplant tolerance (44, 58).

Although therapy with rIL-4 (59, 60) or rIL-13 (61) delayed rejection, in other models rIL-4 promoted rejection (62–64). Further, in adoptive transfer studies allospecific Th2 cells mediate rejection (44, 45). Th2 cytokines are produced during normal rejection where there is induction of Th2 cells that contribute to normal allograft rejection responses (58, 65).

We concluded that the effects of rIL-5 in this study are attributed to its role in activation of Type-2 Treg. IL-5 is a cytokine produced by Th2 cells and some regulatory T cells including Tr1 and Ts1 cells (29, 66). IL-5 is produced long-term by Th2 cells, after the initial burst of IL-4 production diminishes. IL-5 acts by binding to a specific IL-5 receptor, IL-5Rα, which has limited expression. In man, IL-5Rα is mainly expressed on eosinophils, basophils and mast cells, and their progenitors (67). IL-5Rα is not expressed by human effector T cells including Th1, Th2, Th17 cells, nor APC, monocytes and macrophages (67). Over 30 years ago, IL-5 was reported to act with rIL-2 to induce cytotoxic T cells (68), but this finding has not been reproduced. Until we described IL-5Rα expression on IL-4 and antigen activated Treg and the capacity of IL-5 to promote their proliferation and expansion (21, 22), there was no solid evidence that IL-5 activated any T cells. We showed that human Treg activated by alloantigen and rIL-4 (not rIL-2) also are induced to express IL-5Rα (30)

IL-5 can activate $CD5^+$B1 cells that express IL-5Rα (69) to produce natural IgM antibodies in response to bacterial stimulation (70, 71). IL-5 promotes murine, but not human, B cells to switch to produce non-complement fixing immunoglobulin isotypes IgG1 and IgE (67). rIL-5 therapy in autoimmunity does not induce a switch in Ig isotypes nor reduce Ig titres (30). IL-5, but not IL-4, induces expression of CD25 on activated B cells (72, 73) and leads to release of soluble CD25 (74), which could consume IL-2. The effect of IL-5 on B cells, as well as of anti-CD25 mAb on activated B cells, was not examined. We cannot exclude that B cells activation contributed to the rIL-5 effect on allograft rejection.

The results of this study are consistent with our findings in autoimmune models that rIL-5 therapy reduces immune inflammation (30, 32). The allograft model we used has only a class I MHC and multiple minor incompatibilities (38, 39, 75) making rejection slower than with both a class I and II MHC mismatch. In a neonatal heart transplant model, rIL-5 therapy delayed rejection and inhibited production of IFN-γ and IL-2.

Our findings of accelerated rejection by blocking IL-4 or by depleting $CD25^+$cells are consistent with $CD4^+CD25^+$ Treg in the host being activated to Ts2 cells by alloantigen and the IL-4 produced by the alloantigen-activated effector T cells. In autoimmunity, blocking IL-4 and depleting $CD25^+$ cells also abrogate the ability of rIL-5 to promote Ts2 cells to reduce immune injury (30, 32). In both autoimmunity and allograft rejection, the activation of Treg by Type-2 cytokines reduces inflammation.

In this study, we showed that Ts2 cells re-cultured with the same donor alloantigen and rIL-5 were induced to express mRNA for the Th2 transcription factors *Gata3* and *Irf4*, together with *il5*. Thus, *in vitro* we showed induction of Th2-like Treg. The $CD4^+CD25^+$T cells from rats with an allograft that had been treated with rIL-5 long-term had cells with a Th2-like Treg phenotype, in that they expressed *Foxp3, Irf4* and *Il5*. IRF4 is a transcription factor that is induced by TCR activation by antigen (76, 77) and the activation of a variety of immune cells in a Type 2 response (78) including antigen-activated Treg that control Th2 responses (36, 37). GATA-3 is the master transcription factor for Th2 responses. Further, in this study we showed *in vitro* induced Th2-like Treg expressed mRNA for *Il5*, which is not expressed by naïve Treg or Ts2 cells. The findings in this study were consistent with rIL-5 therapy promoting antigen-specific Treg that include Th2-like Treg.

In our studies in several models of alloimmunity (34) and autoimmunity (30, 32, 79), rIL-5 therapy was well tolerated. Mice with transgenes for IL-5 have high levels of IL-5 and eosinophilia, but remain healthy (38, 39, 80). High levels of IL-5 produced by Th2 responses to parasitic infections induces eosinophilia but has no adverse effects. The impairment of autoimmunity by parasitic infection in part depends on IL-5 and $CD25^+$T cells activated by IL-4 (23).

$CD4^+CD25^+$cells from heart grafted animals treated with rIL-5 for 10 days, at the end of rIL-5 treatment, had increased proliferation to specific donor Lewis that was partially enhanced by addition of rIL-5 to cultures. These cells did not respond to self or third-party stimulator cells, even in the presence of rIL-5.

$CD4^+CD25^+$ cells of animals treated for 10 days with rIL-5, whose allografts survived >60 days, lacked reactivity to specific donor alloantigen unless rIL-5 was present in the cultures. These findings are consistent with an alloantigen-specific response of the tolerant $CD4^+CD25^+$T cells, we have recently described (31). Briefly, $CD4^+CD25^+$T cells from animals tolerant to a graft have no reactivity to donor antigen in the absence of cytokines such as IL-5 (31). Tolerant Type-2 cytokine activated Treg are dependent on IL-5, so cells have no response if rIL-5 is not present. Moreover, in the absence of rIL-5 *in vivo*, the alloantigen-specific Ts2 cells did not survive and other Treg, such as Ts1 and Th1-like Treg were activated. These results suggest that rIL-5 therapy may need to be given long-term to sustain the Ts2 cells and induce Th2-like Treg that express IRF4 and produce IL-5.

The mechanisms by which antigen specific Treg suppress rejection are not fully understood. They can enter the sites of inflammation in the graft, where they neutralize effector responses, including by production of adenosine by CD39 and CD73 expressed by activated Treg (81). Other less well understood mechanisms require direct Treg contact with effector cells that appear to involve Class II MHC on activated Treg and release of perforin and granzyme. Studies of the effector function of activated Treg are complicated by their dependence on specific antigen stimulation and cytokines such as IFN-γ (29, 31, 82) and IL-12 (28, 31) in the case of Type-1 activated Treg, or IL-5 in the case of Type 2 activated Treg (31, 33). Antigen-specific Treg die in culture without the cytokines required to support their survival (31, 83), and do not suppress proliferation of effector T cells *in vitro* (31, 84, 85). This is a distinct difference to naïve/resting tTreg which inhibit antigen presenting cells and reduce activation of naïve effector T cells (25). Given there is no assay for assessing suppression of activated alloantigen specific $CD4^+CD25^+Foxp3^+$Treg *in vitro*, we were unable to assess their function *ex vivo*.

This study showed rIL-5 promoted induction of Treg that inhibited rejection to promote induction of tolerance. The inability of IL-5 to promote effector T cells makes it a better candidate for induction of tolerance than Type-1 cytokines IFN-γ (56, 57, 82, 86, 87) or IL-12 (28, 29) that promote Th1-like Treg but also promote Th1 responses and NK cells. Type-I induced and activated Treg were also generated in rIL-5 treated hosts demonstrating the pathways were complimentary and not mutually exclusive.

Although a variety of regulatory mechanisms have been described to promote transplant tolerance, the dominant regulatory mechanism in most models involves $CD4^+CD25^+$ $Foxp3^+$Treg (16, 17, 23, 31, 43). Human $CD4^+CD25^+$ $CD127^{lo}$Treg activated by rIL-4 and alloantigen express IL-5Rα (27). Thus, therapy with rIL-5 or an analogue may be of use to induce antigen-specific activated $CD4^+CD25^+$ Treg in man and suggests a new pathway to control ongoing rejection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by University of New South Wales Animal Ethic Committee.

AUTHOR CONTRIBUTIONS

BH: Participated in research design, writing of paper, and data analysis. RH: Participated in conduct of research, writing of paper, and data analysis. GT: Participated in conduct of research, writing of paper, and data analysis. CR: Participated in conduct of research, writing of paper, and data analysis. PW: Participated in conduct of research, writing of paper, and data analysis. PR: Participated in conduct of research.CW: Participated in conduct of research. AS: Participated in research design, writing of paper, and data analysis. NV: Participated in research design, conduct of research; writing of paper, and data analysis. SH: Participated in research design, writing of paper, and data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.714838/full#supplementary-material

Supplementary Figure 1 | Photomicrographs (x40) of transplanted Lewis heart grafts in F344 recipients.

Supplementary Figure 2 | Photomicrographs (x40) of transplanted Lewis heart grafts in F344 recipients. Comparison of grafts from sham and rIL-5 treated recipients. Immunoperoxidase staining with monoclonal antibodies to CD4, CD8, Foxp3 and ED1 (a macrophage marker). Quantitation of infiltrate shown in **Figure 4D**.

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Conflict of Interest : BH and SH hold patents related to production of antigen specific Treg and tests of tolerance related to this work.

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Oxidative Stress Promotes Instability of Regulatory T Cells in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis

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We investigated the characteristics of regulatory T cells (Tregs), focusing on the relationship between their stability and reactive oxygen species (ROS), in antineutrophil cytoplasmic antibody-associated vasculitis (AAV). Intracellular expressions of effector cytokines, forkhead box protein 3 (FoxP3), ROS, phosphorylated mammalian target of rapamycin (mTOR), and sirtuin 1 (SIRT1) in Tregs from peripheral blood mononuclear cells (PBMCs) of patients with AAV and healthy controls (HC) were analyzed. The alterations in and functional ability of Tregs were compared before and after resveratrol (RVL) treatment of PBMCs in patients with AAV. Significantly higher expressions of interferon (IFN)-γ, interleukin (IL)-17, IL-4, ROS, and phosphorylated mTOR (pho-mTOR) and lower expression of SIRT1 in CD4+CD25+FoxP3+ cells were found in patients with AAV than in the HC. FoxP3 expression in CD4+CD25+ cells and suppressive function of Tregs were significantly lower in patients with AAV than in the HC. Tregs after RVL treatment demonstrated significant decreases in IFN-γ, ROS, and pho-mTOR levels and increases in FoxP3, SIRT1 levels, and functional activity. Conversely, the direct activation of SIRT1 by SRT1720 resulted in decreased FoxP3 expression, with no reduction in ROS levels. The pho-mTOR levels were significantly higher in Tregs after activation by SRT1720 than in those after RVL treatment. This study suggested that imbalanced changes in Tregs could be attributed to mTOR activation, in which ROS overproduction was predominantly implicated. Therefore, ROS is a key mediator for promoting Tregs instability in AAV.

Keywords: regulatory T cells, reactive oxygen species, FoxP3, ANCA-associated vasculitis, effector cytokines, plasticity, mTOR, resveratrol

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INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a systemic autoimmune disorder involving a pauci-necrotizing small-sized vasculitis, in which ANCA targeting myeloperoxidase (MPO) and proteinase 3 (PR3) participate in the pathogenesis of the disease. Suggestive etiologies, including various environmental factors and multifactorial susceptibility genes, have been shown to date (1). Moreover, divergent pathological mechanisms

in both innate and acquired immune systems are implicated in the development of AAV, suggesting that inflammatory damage of targeted organs involving vasculitis and granuloma formation is promoted by hyperactivated immunocompetent cells, such as neutrophils, macrophages, autoreactive B cells, and T cells, as well as proinflammatory cytokines and reactive oxygen species (ROS) (1, 2). Some studies have also reported the dysfunction and imbalance of regulatory T cells (Tregs) in AAV (3–5). Additionally, the plasticity of Tregs involving a shift to helper T (Th)-like cells by intracellular expression of effector cytokines is induced in inflammatory autoimmune diseases (6, 7). However, it remains uncertain how the plastic changes of Tregs are evoked in AAV, although it has been hypothesized that dysfunction of Tregs might be exerted by exposure to effector cytokines, especially conversion to the Th17-lineage (8). Hyperexpression of Th cells is strongly implicated in the development of AAV, suggesting that the etiologic factors related to AAV could lead to the abrogation of the intracellular signaling of Tregs, such as forkhead box P3 (FoxP3) expression. Oxidative stress adversely affects the expression and functional ability of Tregs in the pathogenic mechanisms of systemic lupus erythematosus (SLE) (9). The plasticity of T cells is physiologically promoted under enhanced oxidative stress; moreover, the expression of effector cytokines may be altered depending on the concentration of ROS (10, 11). Given these immune reactions and the pathogenesis of AAV, it is necessary to consider that oxidative stress may be a mediator affecting the conditions of Tregs because oxidative stress plays a crucial role in the development of AAV (2). The mammalian target of rapamycin (mTOR) pathway can be affected by metabolic alterations, including oxidative stress (12). In the development of Tregs, mTOR activation inhibits FoxP3 expression, which is also associated with the induced expression of effector cytokines in T cells (12). Accordingly, it is necessary to investigate the intracellular circumstances of ROS expression and mTOR activation as clues for promoting the plasticity of Tregs in AAV. It is still uncertain how oxidative stress affects the kinetics of Tregs in AAV.

Herein, we investigated the characteristics of Tregs, focusing on their imbalanced alteration, including expression of effector cytokines, ROS, mTOR activation, and their functional ability. Additionally, we evaluated the stability of Tregs after treatment with resveratrol (RVL), a phenolic compound that can potentially exert antioxidant, anti-immune aging, and anti-inflammatory effects (13, 14). RVL is also known as a potential activator of sirtuin (silent mating type information regulating 2 homolog) 1 (SIRT1), which is a nicotinamide adenosine dinucleotide $(NAD)^+$-dependent histone/protein deacetylase that serves as a substrate for stabilizing mammalian physical functions (15). SIRT1 also regulates inflammatory and metabolic reactions of immunocompetent cells as an anti-immune aging and homeostasis mediator (16, 17). RVL may be an anti-aging therapy. Therefore, we evaluated SIRT1 expression in Tregs. To the best of our knowledge, this is the first attempt to investigate the characteristics of circulating Tregs, focusing on their plasticity and oxidative damage in AAV.

MATERIAL AND METHODS

Patients

Twenty-five patients with microscopic polyangiitis (MPA) or granulomatosis with polyangiitis (GPA) who had not received immunosuppressive therapy were enrolled in this study. The diagnosis and classification of MPA or GPA were determined according to the criteria of the Chapel Hill Consensus Conference (18) and/or the consensus algorithm proposed by the European Medicines Agency (19). Patients with complications of neoplasms or infections were excluded from the study. Of the 25 patients (mean age, 63 years; 8 men and 17 women), 14 (56%) and 11 (44%) were classified as MPA and GPA, respectively. The Birmingham Vasculitis Activity Score (BVAS) (20) was 19.2 ± 6.7. The related symptoms based on BVAS and laboratory findings, which included white blood cell count, serum levels of C-reactive protein, erythrocyte sedimentation rate, and presence of MPO-ANCA or PR3-ANCA, were also evaluated before initiating treatment (**Supplementary Table 2**). For comparison, 17 age-matched healthy controls (HC), with a mean age of 58 years (seven men, 10 women), were included in the control group. Whole blood samples were obtained from 25 patients prior to initiating immunosuppressive therapy and 17 HC enrolled in this study. The local Ethics Committee of Shinshu University approved this study (approval number: 614). All participants provided written informed consent.

Cell Isolation and Quantitative Real-Time Polymerase Chain Reaction

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples collected in EDTA-coated tubes by gradient centrifugation with Ficoll-Hypaque PLUS (GE Healthcare, Pittsburgh, PA, USA). The CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) isolated Tregs from unstimulated PBMCs. Total RNA was extracted from isolated Tregs using an RNeasy Mini kit (Qiagen, Venlo, Netherlands). Complementary DNA (cDNA) was synthesized using the Maxima First-Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). cDNA was used to perform qRT-PCR with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (Takara, Kusatsu, Japan). The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (QT00079247 [Hs_GAPDH_1_SG]), FoxP3 (QT00048286 [Hs_FoxP3_1_SG]) (both from Qiagen), and mTOR (qHsaCID0012480 [ENSG00000198793]) (Bio-Rad, Hercules, CA, USA). In order to evaluate the results in qRT-PCR quantitatively, relative copy number (RCN) was calculated using threshold cycle (Ct) of GAPDH and target gene as follows: ΔCt = Ct (target gene) – Ct (GAPDH); $RCN = 2^{-\Delta Ct}$.

Cell Treatment and Flow Cytometry

Isolated PBMCs were incubated on a 24-well plate (1×10^6/well) with and without 100 μM RVL (Sigma-Aldrich, St. Louis, MO, USA) or 5 μM SRT1720 (Abcam, Cambridge, UK) at 37 ° for 24 h.

Incubated PBMCs were stimulated with 0.5 μg/ml ionomycin, 0.04 μg/ml phorbol myristate acetate (both from Sigma-Aldrich), and 2 μM monensin (BD Biosciences, San Diego, CA, USA) at 37° C for 4 h. Stimulated PBMCs were stained with PE/Cy7-conjugated anti-CD4 (BioLegend, San Diego, CA, USA) and PC5-conjugated anti-CD25 (Beckman Coulter, Brea, CA, USA) with or without PE-conjugated anti-CD152 (cytotoxic T-lymphocyte-associated protein 4, CTLA-4) (Beckman Coulter) antibodies. The stained PBMCs were permeabilized with Cytofix/Cytoperm (BD Biosciences) and then stained with FITC-conjugated anti-IFN-γ (Beckman Coulter), PE-conjugated anti-IL-17 (BD Biosciences), PE-conjugated anti-IL-4 (Beckman Coulter), FITC-conjugated transforming growth factor (TGF)-β1 (BioLegend), PE-conjugated IL-10 (BioLegend) antibodies, or PE-conjugated mTOR (pS2448) antibody (BD Biosciences) for detecting phosphorylated mTOR (pho-mTOR), as well as PE-conjugated (BD Biosciences), FITC-conjugated, or Pacific blue-conjugated anti-FoxP3 (both from BioLegend). Alternatively, permeabilized PBMCs were stained with Alexa Fluor 405-conjugated anti-SIRT1 (Novus Biologicals, Littleton, CO, USA) and PE-conjugated anti-FoxP3. Treated cells were acquired on a FACSCanto II flow cytometer (BD Bioscience), and the data were analyzed using FlowJo software version 7.6.5 (Tree Star Inc., Ashland, OR, USA).

Measurement of Intracellular ROS

PBMCs incubated with and without RVL or SRT1720 were stimulated with 200 μM tert-butyl hydroperoxide at 37 °C for 60 min. Treated PBMCs were fixed and permeabilized using FoxP3-staining buffer set (BD Bioscience) after staining with CellROX Deep Red Reagent (Invitrogen, Carlsbad, CA, USA), PE/Cy7-conjugated anti-CD4, and PC5-conjugated anti-CD25 antibodies. Permeabilized PBMCs were stained with PE-conjugated FoxP3. Intracellular ROS was detected on a flow cytometer and analyzed using FlowJo software.

Suppression Assay of Tregs

Suppression assays were performed to evaluate the suppressive ability of Tregs. Tregs were isolated from incubated PBMCs with or without RVL using the CD4+CD25+ regulatory T cell isolation kit. To detect the target cells for Tregs, conventional T (Con-T) cells (CD4+CD25- cells) were isolated from untreated PBMCs of HC. Allogenic Con-T cells labeled with carboxyfluorescein succinimidyl ester (CFSE; 2μM, Invitrogen) and Tregs were co-cultured with anti-CD3/CD28 microbeads (Invitrogen) at a ratio of 1:1:1 in a 96-well U-bottom plate at 37°C for 4 d. The proliferation of Con-T cells was determined by CSFE dilution, and was acquired on a flow cytometer. The data were analyzed using FlowJo software.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). The Mann-Whitney U test and Fisher's exact probability test were used to compare the two independent groups. Consecutive data with and without treatment were compared using the Wilcoxon signed-rank test. The Kruskal-Wallis test was performed for comparisons among three independent groups, and the Steel-Dwass test was used for multiple comparisons. Statistical significance was defined as a *p-value* less than 0.05. All statistical analyses were performed using BellCurve for Excel (SSRI, Tokyo, Japan).

RESULTS

Frequency of Tregs and Their Intracellular Expression of Effector Cytokines in AAV

The percentage frequency of circulating Tregs (CD4+CD25+FoxP3+ cells) was significantly lower in the patients with AAV than in the HC (p = 0.0004) (**Table 1**). FoxP3 expression in CD4+CD25+ population and relative copy number (RCN) of FoxP3 in isolated Tregs were significantly lower in the patients with AAV than in the HC (p <0.0001, p = 0.040, respectively) (**Figures 1A–C**). Intracellular expression of IFN-γ, IL-17, and IL-4 in Tregs was significantly higher in the patients with AAV than that in the HC (median fluorescence index [MFI]: p < 0.0001, p = 0.0003, p = 0.0009, respectively) (**Figure 1D**) (frequency: p = 0.002, p = 0.032, p = 0.004, respectively) (**Table 2**). The percent frequencies of IFN-γ, IL-17, and IL-4 positive CD4+CD25+FoxP3+ cells were significantly higher in the patients with AAV than in the HC (p = 0.009, p = 0.039, p = 0.008, respectively) (**Figure 1E**). In the additional analyses of CD4+CD25+CD127-/lowCD45RA+FoxP3+ cells, the percent frequency of them was significant lower in the patients with AAV than the HC (p = 0.025) (**Supplementary Table 3**). FoxP3 expression in CD4+CD25+CD127-/lowCD45RA+ cells was also significantly lower in the patients with AAV than in the HC (p = 0.0001), and expression of effector cytokines, including IFN-γ, IL-17, and IL-4, in CD4+CD25+CD127-/lowCD45RA+FoxP3+ cells were significantly higher in patients with AAV than in the HC (MFI: p < 0.0001, p = 0.003, p < 0.0001, respectively) (**Supplementary Figure 1**) (frequency: p < 0.0001) (**Supplementary Table 3**). Meanwhile, in comparison of intracellular expression of IFN-γ, IL-17, and IL-4 in high-and low-density expression of FoxP3 in the patients with AAV, their expression were significantly higher in the population of FoxP3high than in that of FoxP3low (p = 0.0001) (**Supplementary Figure 2**). In the HC, intracellular expression of IFN-γ, IL-17, and IL-4 was not significantly different in two distinct population of FoxP3 (p = 0.280, p = 0.306, p = 0.864, respectively).

Accordingly, these results suggested that significant increases in intracellular expression of effector cytokines and decreased FoxP3 expression are characteristics of Tregs in patients with AAV.

Intracellular Mediators Affecting Plasticity of Tregs in AAV

ROS production in Tregs was significantly greater in the patients with AAV than in the HC (p <0.0001) (**Figure 2A**). The RCN of mTOR in isolated Tregs was not significantly different between the patients with AAV and the HC (p = 0.828) (**Figure 2B**), whereas expression of pho-mTOR in Tregs was significantly

TABLE 1 | Frequency of CD4+CD25+FoxP3+ cells in patients with AAV and healthy controls.

	AAV (n = 25)	HC (n = 17)	*p*-value
In total lymphocytes			
% CD4+ cells	48.99 ± 18.09	47.41 ± 9.09	0.682
% CD4+CD25+ cells	21.19 ± 13.35	21.46 ± 11.57	0.729
% CD4+CD25+FoxP3+ cells	5.34 ± 3.83	10.68 ± 6.76	0.0004

AAV, ANCA-associated vasculitis; HC, healthy controls. Data are presented as the mean ± SD.

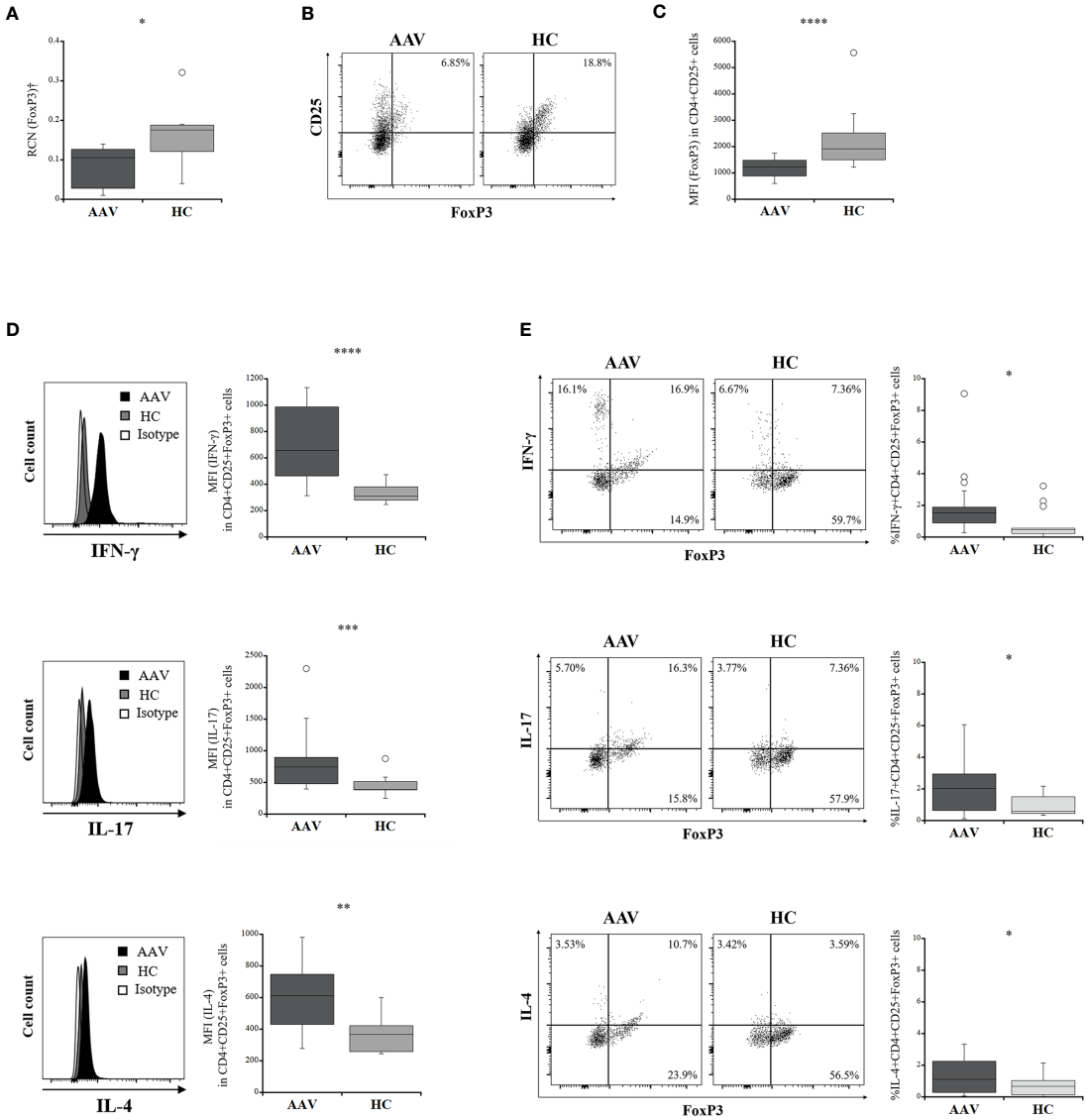

FIGURE 1 | Comparisons of intracellular expression of FoxP3 and effector cytokine levels in regulatory T cells (Tregs) between the patients with ANCA-associated vasculitis (AAV) and healthy controls (HC). **(A)** relative copy number (RCN) of FoxP3 in isolated Tregs (AAV, n = 10; HC, n = 7). **(B)** representative dot plots showing CD25+FoxP3+ in CD4+ cells. **(C)** the median fluorescence index (MFI) of FoxP3 in CD4+CD25+ cells (AAV, n = 18; HC, n = 15). **(D)** representative histograms showing expression of interferon-γ (IFN-γ), interleukin (IL)-17, or IL-4 in CD4+CD25+FoxP3+ cells (left). MFI of IFN-γ, IL-17, or IL-4 in CD4+CD25+FoxP3+ cells (AAV, n = 18; HC, n = 15) (right). **(E)** representative dot plots showing expression of FoxP3 and interferon-γ (IFN-γ), interleukin (IL)-17, or IL-4 in CD4+CD25+ cells (left). Percent frequencies of IFN-γ, IL-17, or IL-4 positive CD4+CD25+FoxP3+ cells (AAV, n = 18; HC, n = 15) (right). †Isolated Tregs from peripheral blood mononuclear cells. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$.

TABLE 2 | Frequencies of intracellular cytokines in Tregs in patients with AAV and healthy controls.

		AAV (n = 18)	HC (n = 15)	*p* value
In CD4+CD25+FoxP3+ cells				
	%IFN-γ	31.09 ± 23.34	8.38 ± 10.41	0.002
	%IL-17	35.05 ± 30.92	9.85 ± 14.33	0.032
	%IL-4	21.54 ± 21.83	2.47 ± 2.44	0.004

Tregs, regulatory T cells; AAV, antineutrophil cytoplasmic antibody-associated vasculitis; HC, healthy controls; IFN-γ, interferon-γ; IL, interleukin.
Data are presented as mean ± SD.

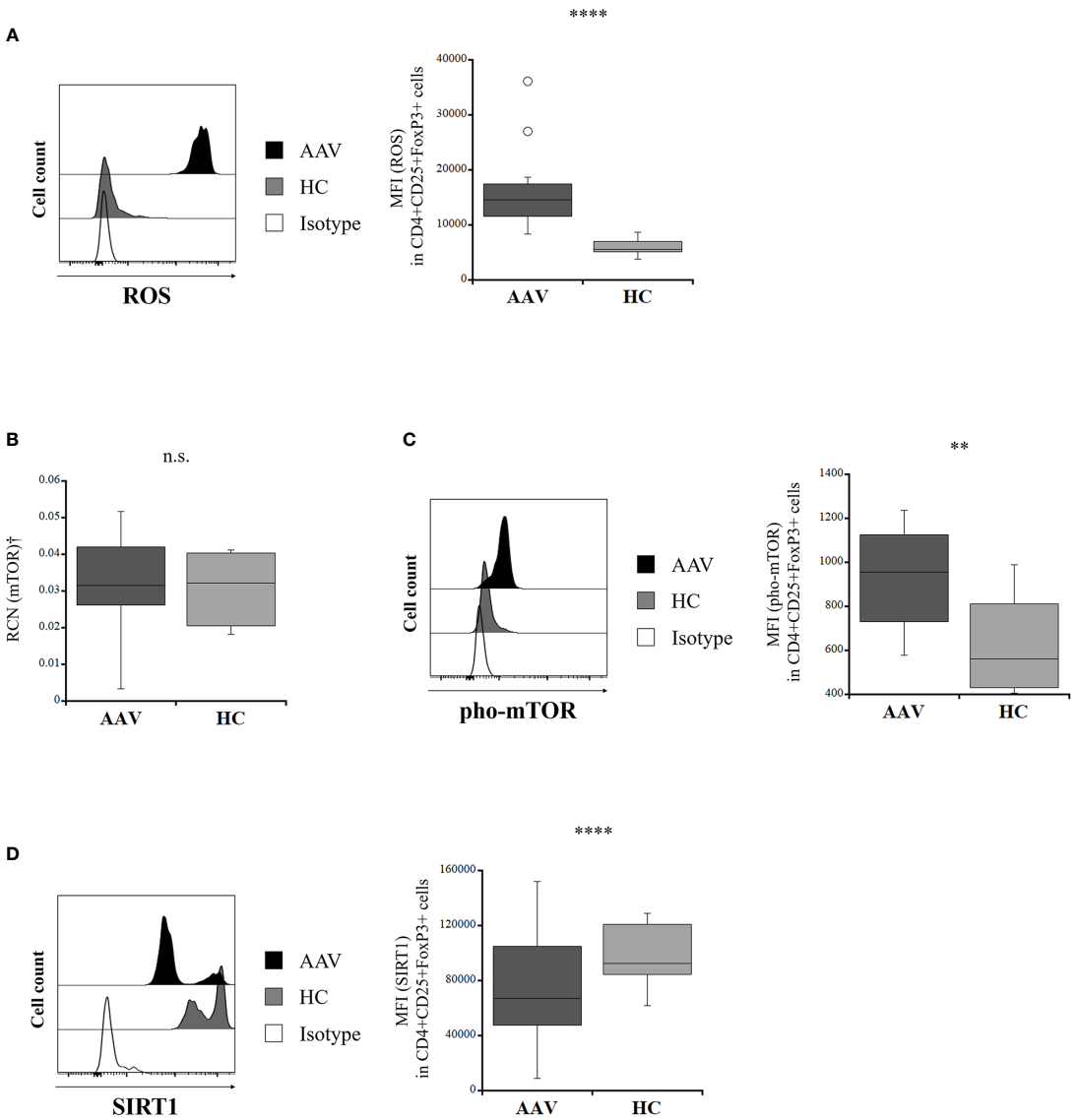

FIGURE 2 | Comparison of intracellular mediators in regulatory T cells between the patients with ANCA-associated vasculitis (AAV) and healthy controls (HC). **(A)** representative histogram showing expression of reactive oxygen species (ROS) in CD4+CD25+FoxP3+ cells (left). The median fluorescence index (MFI) of ROS in CD4+CD25+FoxP3+ cells (AAV, n = 20; HC, n = 13) (right). **(B)** relative copy number (RCN) of mammalian target of rapamycin (mTOR) in isolated Tregs (AAV, n = 10; HC, n = 7). **(C)** representative histogram showing expression of phosphorylated mTOR (pho-mTOR) in CD4+CD25+FoxP3+ cells (left). MFI of pho-mTOR in CD4+CD25+FoxP3+ cells (AAV, n = 18; HC, n = 12) (right). **(D)** representative histogram showing expression of sirtuin 1 (SIRT1) in CD4+CD25+FoxP3+ cells (left). MFI of SIRT1 in CD4+CD25+FoxP3+ cells (AAV, n = 23; HC, n = 14) (right). †Isolated Tregs from peripheral blood mononuclear cells. n.s., not significant; $^{**}p < 0.005$; $^{****}p < 0.0001$.

higher in the patients with AAV than that in the HC ($p = 0.003$) (**Figure 2C**). SIRT1 expression was significantly lower in the patients with AAV than in the HC ($p < 0.0001$) (**Figure 2D**).

Changes in the Intracellular Environment in Tregs After Treatment With RVL

We evaluated the intracellular expression of etiologic factors described above in Tregs with and without RVL treatment in the patients with AAV. IFN-γ expression was significantly decreased in Tregs after RVL treatment ($p = 0.003$) (**Figure 3**), but was significantly higher than in the HC ($p = 0.0001$). When comparing IL-17 and IL-4 expression in Tregs, there was no significant difference with or without RVL treatment. The percent frequencies of IFN-γ, IL-17, and IL-4 positive CD4+CD25+FoxP3+ cells with and without RVL treatment were not significantly different in the patients with AAV ($p = 0.132$, $p = 0.214$, $p = 0.325$, respectively) (**Supplementary Figure 3**). The expression of FoxP3 in CD4+CD25+ cells was significantly increased after RVL

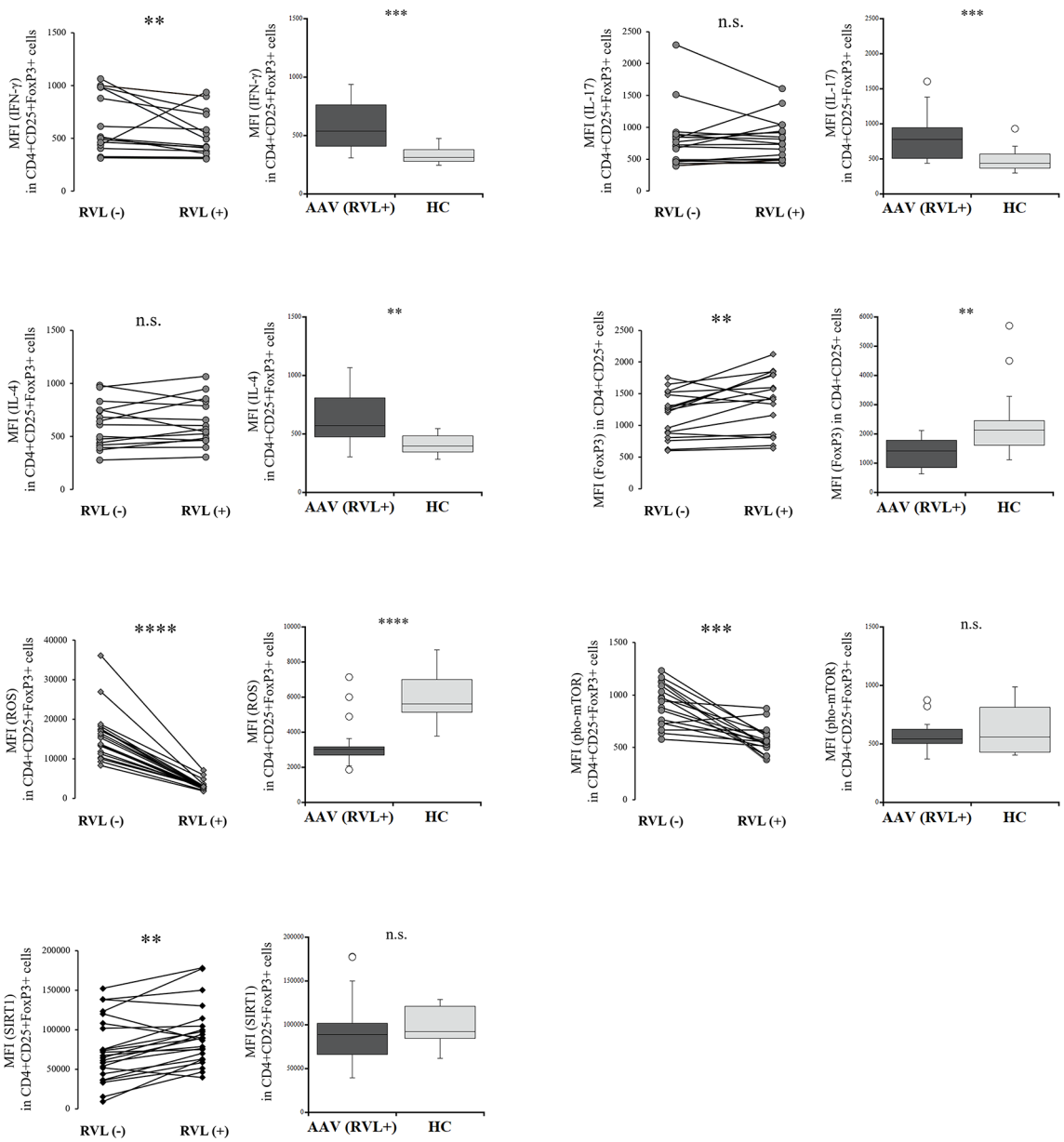

FIGURE 3 | Comparisons of regulatory T cells with and without resveratrol (RVL) treatment in the patients with AAV. Comparisons with and without RVL treatment (left) and those between after RVL treatment in the patients with AAV and healthy controls (HC) (right), with regards to median fluorescence index (MFI) of interferon-γ (IFN-γ), interleukin (IL)-17, or IL-4 in CD4+CD25+FoxP3+ cells (n = 18), MFI of FoxP3 in CD4+CD25+FoxP3+ cells (n = 18), MFI of ROS in CD4+CD25+FoxP3+ cells (n = 20), MFI of phosphorylated mammalian target of rapamycin (pho-mTOR) in CD4+CD25+FoxP3+ cells (n = 18), and MFI of sirtuin 1 (SIRT1) in CD4+CD25+FoxP3+ cells (n = 23). n.s., not significant; $^{**}p < 0.005$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

treatment ($p = 0.002$), but was lower than in the HC ($p = 0.002$). The production of ROS in Tregs was significantly decreased after RVL treatment ($p < 0.0001$), and was significantly lower than that in the HC ($p < 0.0001$). Additionally, pho-mTOR expression in Tregs was also significantly decreased after RVL treatment ($p = 0.0006$), but the levels were not significantly different from those in the HC ($p = 0.525$). The expression of SIRT1 in Tregs was significantly increased after RVL treatment ($p = 0.002$). In the HC, decreased expression of ROS and pho-mTOR and increased expression of SIRT1 were significantly shown in CD4+CD25+FoxP3+ cells with RVL treatment ($p = 0.001$, $p = 0.002$, $p = 0.008$, respectively), whereas intracellular expression of IFN-γ, IL-17, IL-4, and FoxP3 was not significantly different (**Supplementary Figure 4**).

Hence, RVL could contribute to decreasing ROS expression and phosphorylation of mTOR, as well as increase in SIRT1, in Tregs from the patients with AAV and HC. In addition, decreased expression of IFN-γ in Tregs and their increased expression of FoxP3 after RVL treatment were also demonstrated in the patients with AAV.

Comparison of the Intracellular Environment in Tregs With and Without SRT1720

To determine the differences in Tregs treated with RVL from those in which SIRT1 was selectively activated, we evaluated the intracellular expression of etiologic factors in Tregs with and without SRT1720 treatment. The expression of IFN-γ and IL-4 was significantly decreased in Tregs after SRT1720 treatment ($p = 0.003$ and $p = 0.013$, respectively) (**Figure 4**), but both IFN-γ and IL-4 levels were not significantly different in Tregs treated with SRT1720 compared to those treated with RVL ($p = 0.393$ and $p = 0.068$, respectively). IL-17 levels were not significantly different between Tregs with and without SRT1720 treatment ($p = 0.182$). The percent frequency of IFN-γ+CD4+CD25+FoxP3+ cells was significantly decreased after SRT1720 treatment in patients with AAV ($p = 0.026$), whereas those of IL-17 and IL-4 positive CD4+CD25+FoxP3+ cells with and without SRT1720 treatment were not significantly different in the patients with AAV ($p = 0.423$, $p = 0.200$) (**Supplementary Figure 3**). Meanwhile, the expression of FoxP3 was significantly lower in CD4+CD25+ cells with SRT1720 than that in the cells without SRT1720 treatment ($p = 0.003$). ROS expression in Tregs was not significantly different with or without SRT1720 treatment ($p = 0.182$). The expression of pho-mTOR was significantly decreased in Tregs after SRT1720 treatment ($p = 0.003$), but expression levels of pho-mTOR were significantly higher in Tregs treated with SRT1720 than those in Tregs treated with RVL ($p < 0.0001$). In the HC, decreased expression of IL-4 and pho-mTOR and increased expression of SIRT1 were significantly demonstrated in CD4+CD25+FoxP3+ cells with SRT1720 treatment ($p = 0.013$, $p = 0.005$, $p = 0.005$, respectively), no significant differences in intracellular expression of IFN-γ, IL-17, ROS, and FoxP3 (**Supplementary Figure 4**).

Consequently, SRT1720 could contribute to decreasing phosphorylation of mTOR and increasing IL-4 and SIRT1 in Tregs from the patients with AAV and HC. In the patients with AAV, decreased expression of IFN-γ was also demonstrated in Tregs with SRT1720 treatment. However, Tregs with SRT1720 treatment significantly demonstrated decreased expression of FoxP3; meanwhile, they had no efficacy in reducing ROS.

Intracellular Expression of IFN-γ, IL-17, and IL-4 in CD4+CD25- Cells and Their Changes With and Without RVL or SRT1720 Treatment

The expression of IFN-γ, IL-17, and IL-4 in CD4+CD25- cells was significantly higher in the patients with AAV than in the HC ($p < 0.0001$, $p = 0.0019$, $p = 0.0009$, respectively) (**Supplementary Figure 5**). In the patients with AAV, IFN-γ expression was significantly decreased in CD4+CD25- cells after RVL and SRT1720 treatment ($p = 0.016$, $p = 0.041$). However, IFN-γ expression in CD4+CD25- cells after RVL and SRT1720 treatment were significantly higher than that in the HC ($p = 0.0006$, $p = 0.0002$).

Suppression Ability of Tregs With and Without RVL Treatment

The proliferation of con-T target cells was evaluated to determine the suppressive ability of Tregs. The proliferation of con-T cells in the presence of Tregs from the HC was significantly lower than that in the absence of Tregs ($p = 0.0001$) (**Figures 5A, B**). The proliferation of con-T cells in the presence of Tregs from the patients with AAV was significantly higher than that in the presence of Tregs from the HC ($p = 0.0047$), demonstrating that the suppressive function of Tregs is impaired in AAV, although the proliferation of con-T cells in the presence of Tregs from AAV was significantly lower than that in the absence of Tregs ($p = 0.016$). Comparing the suppressive ability of Tregs with and without RVL treatment, the proliferation of con-T cells in the presence of Tregs treated with RVL was significantly lower than that in Tregs without RVL ($p = 0.017$) (**Figure 5C**). The proliferation of con-T cells in the presence of Tregs treated with RVL was higher, although not significantly different from that in the presence of Tregs from the HC ($p = 0.612$) (**Figure 5B**).

The expression of co-inhibitory factors of Tregs, including TGF-β1, IL-10, and CTLA-4, was significantly lower in the patients with AAV than in the HC ($p = 0.0002$, $p = 0.041$, $p = 0.025$, respectively) (**Figure 5D**). After treatment with RVL, the expression of IL-10 and CTLA-4 was significantly increased ($p = 0.002$ and $p = 0.002$, respectively), whereas that of TGF-β1 was not significantly different ($p = 0.059$) (**Figure 5E**).

Taken together, the suppressive ability of Tregs and their expression of co-inhibitory factors were significantly lower in the patients with AAV than in the HC. RVL treatment could significantly increase the suppressive ability of Tregs and their expression of IL-10 and CTLA-4. However, no significant finding of TGF-β1 expression was demonstrated in Tregs after RVL treatment.

DISCUSSION

This study demonstrated a decrease in Tregs together with impaired functional activity in the acute phase of AAV. Some

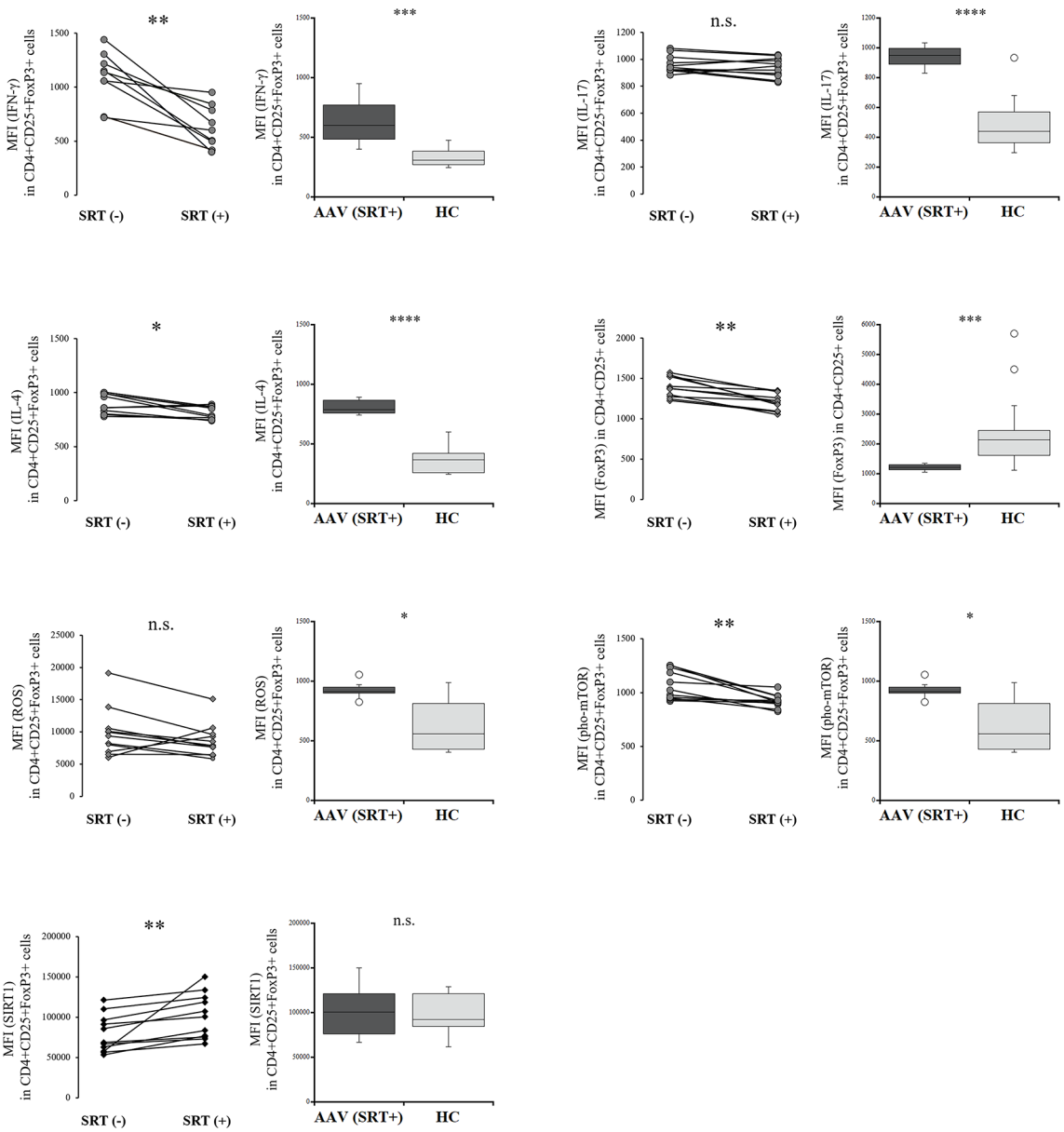

FIGURE 4 | Comparisons of regulatory T cells with and without SRT1720 treatment in the patients with AAV. Comparisons with and without SRT1720 treatment (left) and those between after SRT1720 treatment in the patients with AAV and healthy controls (HC) (right), with regards to median fluorescence index (MFI) of interferon-γ (IFN-γ), interleukin (IL)-17, or IL-4 in CD4+CD25+FoxP3+ cells (n = 11), MFI of FoxP3 in CD4+CD25+FoxP3+ cells (n = 11), MFI of ROS in CD4+CD25+FoxP3+ cells (n = 11), MFI of phosphorylated mammalian target of rapamycin (pho-mTOR) in CD4+CD25+FoxP3+ cells (n = 11), and MFI of sirtuin 1 (SIRT1) in CD4+CD25+FoxP3+ cells (n = 11). n.s., not significant; *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$.

investigations inconsistently indicated decreased, increased, or equivalent expression of Tregs in autoimmune diseases, including AAV, compared to the healthy individuals, whereas dysfunction of Tregs was consistently described (3–5, 7, 21). However, we confirmed imbalanced homeostatic changes in Tregs, in which overexpression of effector cytokines and decreased FoxP3 expression were observed. Furthermore, intracellular increases in oxidative stress and mTOR activation were also demonstrated, suggesting an underlying mechanism for the plasticity and instability of Tregs in AAV. We also assessed how a reduction in SIRT1 is associated with the alterations in Tregs.

The expression of effector cytokines, including IFN-γ, IL-17, and IL-4, was significantly higher in Tregs from the patients with AAV, resulting in plastic changes in Tregs, leading to impaired immune tolerance in the acute phase of AAV. The plasticity of Tregs, which represents effector cytokine expression in the phenotypical population of T cells expressing FoxP3 (6, 7, 22, 23),

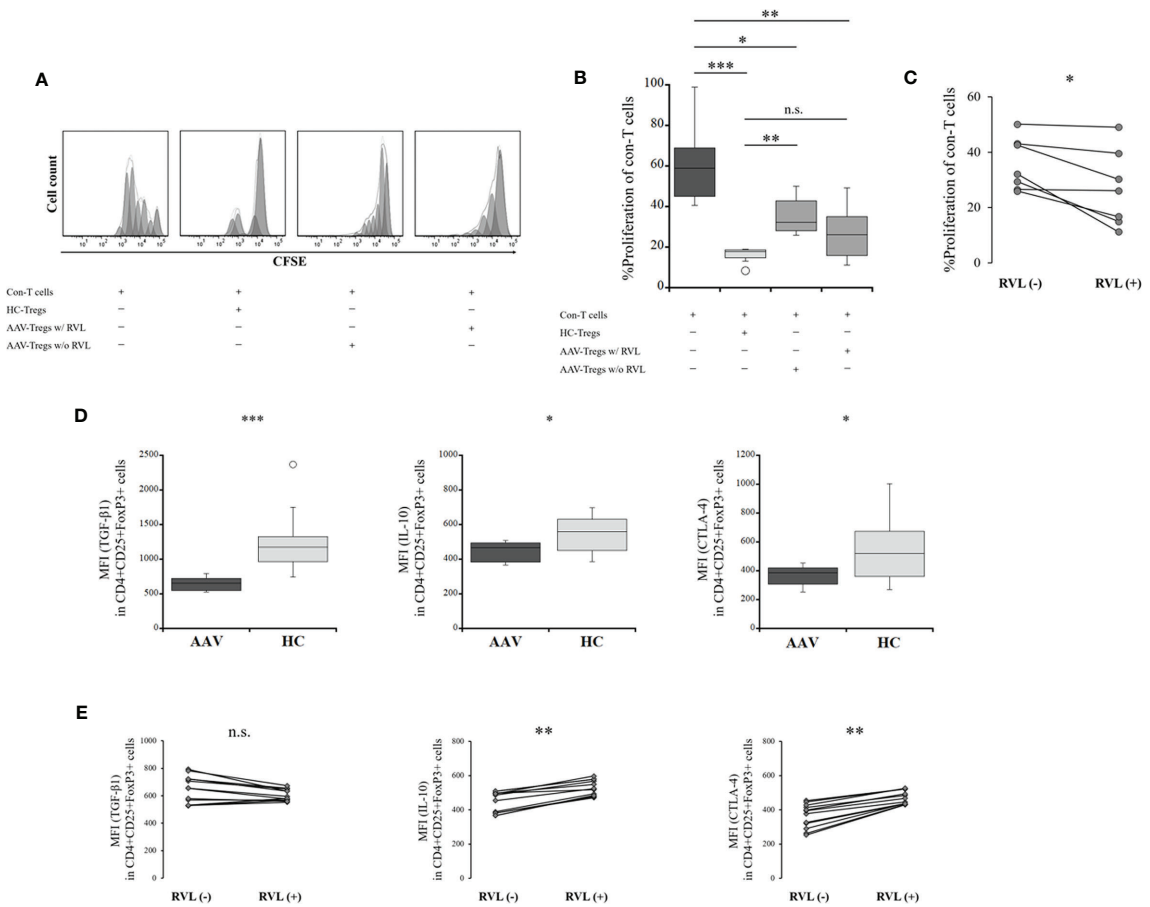

FIGURE 5 | Suppressive assay of regulatory T cells (Tregs) and their co-inhibitory factors before and after treatment with resveratrol (RVL). **(A)** representative histograms showing proliferation of conventional T (Con-T) cells in the absence of Tregs, in the presence of Tregs from the HC (HC-Tregs), in the presence of Tregs from the patients with AAV (AAV-Tregs) with (w/) RVL treatment, and in the presence of AAV-Tregs without (w/o) RVL treatment. **(B)** comparisons of the percent (%) proliferation of Con-T cells in the absence of Tregs (n = 12), in the presence of HC-Tregs (n = 9), AAV-Tregs with RVL (n = 7), and AAV-Tregs without RVL treatment (n = 7). **(C)** alteration of the percent (%) proliferation of Con-T cells with AAV-Tregs before and after RVL treatment (n = 7). **(D)** comparisons of median fluorescence index (MFI) of transforming growth factor (TGF)-β1, IL-10, or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in CD4+CD25+FoxP3+ cells between patients with AAV (n = 12) and HC (n = 10). **(E)** alteration of MFI of TGF-β1, IL-10, or CTLA-4 in Tregs from patients with AAV before and after RVL treatment (n = 12). AAV, ANCA-associated vasculitis; HC, healthy controls; n.s., not significant; $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

could be implicated in insufficient suppressive function (6). The expression of FoxP3 in CD4+CD25+ cells was also decreased in AAV, resulting in Tregs failing in their suppressive ability because FoxP3 expression in the conventional phenotype of Tregs plays a pivotal role in regulating extraordinary immune reactions (24, 25). Additionally, downregulation of FoxP3 could be promoted in response to inflammatory signals (26–28); notably, intracellular induction of effector cytokines, including IFN-γ, IL-17, and IL-4, downregulated FoxP3 expression in Tregs (22, 27, 29). Furthermore, Tregs deficient in FoxP3 obtained effector cell function and lost their suppressive ability (23). We postulated that Tregs in AAV, which were diverted into Th-like Tregs, could ultimately lose FoxP3 expression, resulting in diminished suppressive function. In the suppression assay, we evaluated the functional ability of Tregs isolated using a commercially available magnetic isolation kit. Expression of FoxP3 in isolated Tregs was significantly lower in the patients with AAV than that in the HC. Therefore, the defective Tregs function in AAV could be attributable to decreased FoxP3 expression, which could underlie the conversion to Th-like Tregs. Moreover, the plasticity of Tregs could contribute to insufficient immunological tolerance, leading to disease development.

ROS overproduction in Tregs was also a notable result. Excessive oxidative stress is implicated in the pathogenesis of AAV, wherein activated neutrophils release ROS (2), whereas no evidence of Tregs expressing ROS in the immune system underlying the AAV development has been shown to date. Oxidative stress is diversely implicated in the function of the immunocompetent cells (30). Modest levels of ROS are physiologically necessary for immune cell survival; however, high exposure to ROS could negatively impact immunocompetent cell function and activity (10). It has been suggested that the functional ability of Tregs could be dependent

on the concentration of ROS, or oxidative stress has been implicated in the activation of cofactors related to Treg suppressive ability (31, 32). Furthermore, plastic alteration of the intracellular environment could also be evoked, depending on ROS concentration (10, 11). In an investigation of type I diabetes, induction of oxidative stress reduced Tregs despite inverse induction in cytotoxic T cells (33). Decreased expression of FoxP3, increased expression of effector cytokines, and abrogated suppressive function were also demonstrated together with significantly higher levels of ROS in Tregs from the patients with AAV, suggesting ROS expression could promote the disability and instability of Tregs in AAV.

Oxidative stress also induces the activation of the mTOR pathway within immunocompetent cells *via* Rheb enhancement (12, 34). The expression of pho-mTOR was significantly higher in Tregs from the patients with AAV. mTOR signaling plays a crucial role in regulating the activity of immunocompetent cells, such as protein synthesis, metabolism, proliferation, growth, and survival as a serine/threonine protein kinase. mTOR signaling activation has also been implicated in inducing the transcription of effector cytokines (12, 35). Moreover, persistent activation of mTOR signaling robustly attenuates the function of Tregs (36). Therefore, mTOR may be a key mediator in determining Treg ability and plasticity in AAV. Conversely, inhibition of the mTOR pathway is required for retaining functional ability and FoxP3 expression of Tregs, while preventing the generation of effector T cells (37, 38). Besides, SIRT1 was significantly decreased in Tregs in AAV, suggesting that SIRT1 deficiency could elicit the instability of Tregs. SIRT1 is also necessary for retaining circulating T cell tolerance (39), and SIRT1 acts as an immune-aging modulator (17). Our results verified a significant reduction in ROS and pho-mTOR levels and increased SIRT1 expression in Tregs after RVL treatment. Additionally, increases in FoxP3 expression and the suppressive function of Tregs were also demonstrated. Herein, we needed to investigate whether selective activation of SIRT1 could also provide same efficacies for retaining homeostatic environment in Tregs. However, selective activation of SIRT1 by treatment with SRT1720, known as the direct activator of SIRT1 (14), ultimately resulted in reduced FoxP3 expression in our study. Previous studies have demonstrated the downregulation of FoxP3 by directly activating SIRT1 (40, 41), supporting our experimental results. Moreover, Tregs treated with SRT1720 showed significant overproduction of ROS and lesser reduction in pho-mTOR levels than those treated with RVL. The current results demonstrated that efficient redox homeostasis provides more potent suppression of mTOR phosphorylation than direct SIRT1 activation. RVL indirectly affects the activation of SIRT1; namely, RVL stimulates AMPK, which increases SIRT1 activity by activating the upstream kinase of AMPK, known as LKB1 (14, 42, 43). SIRT1 could also activate AMPK by interacting with LKB1, even in the absence of RVL (43). AMPK is a negative regulator of mTOR (13), suggesting that the regulation of mTOR by RVL treatment or SIRT1 activation could be mediated through AMPK activation. Metformin, which is used to treat diabetes mellitus, also plays a role in negatively regulating mTOR activation by stimulating AMPK (44). NAC (*N*-acetylcysteine), also known as an antioxidant, improved disease activity by suppressing mTOR activation in SLE (45, 46). Moreover, it explained that NAC suppresses induction of ROS, indirectly resulting in a reduction in mTOR activation. Accordingly, RVL may provide integrated roles of negatively regulating ROS-mediated and AMPK signaling, such as a combination of NAC and metformin, in repressing mTOR activation within Tregs from the patients with AAV. Taken together, the instability of Tregs in AAV could be promoted by induced phosphorylation of mTOR, in which intracellular overproduction of ROS could be more prominently implicated than reduced SIRT1 activity.

There were some limitations to this study. RVL may possess the antioxidant ability and suppress mTOR activation, leading to increased FoxP3 expression and functional ability in Tregs. However, the stability of Tregs after RVL treatment in patients was less than that in HC. Plastic changes in Tregs exhibiting increases in IFN-γ, IL-17, and IL-4 expression remained, suggesting that a fundamental immunosuppressive therapy could be required to suppress the inflammatory signal promoting the Th-like shift. Hence, RVL could be useful as adjuvant therapy in partial remission after administering immunosuppressive drugs. Deficient expression of TGF-β1, IL-10, and CTLA-4, which are known as potential mediators that facilitate the suppressive ability of Tregs (21), was also observed in Tregs from the patients with AAV. The expression of these co-inhibitory mediators of Tregs could be negatively regulated by activating mTOR signaling (47). Besides, TGF-β1 promotes the downregulation of mTOR signaling and contributes to maintaining the stability of Tregs and FoxP3 expression (48). Intracellular production of TGF-β1 and CTLA-4 is implicated in retaining Treg ability, whereas the process of TGF-β1 production differed from that of CTLA-4 (47, 49). Tregs ultimately showed a significant increase in the expression of IL-10 and CTLA-4 after RVL treatment, despite not being significantly different from that of TGF-β1, suggesting that the efficacy of RVL has a limitation especially for treating TGF-β1 expression in Tregs. Therefore, further studies are required to investigate the mechanism underlying the stabilization of Tregs. Besides, this study only evaluated efficacies of RVL *in vitro*. Therefore, it is also necessary to perform the clinical research, in which RVL will be administered to the patients with AAV, to develop this attempt for practical application.

In conclusion, Tregs from the patients with AAV showed increased IFN-γ, IL-17, and IL-4 expression, decreased FoxP3 expression, and impaired functional activity. Imbalanced changes in Tregs could be attributed to induced phosphorylation of mTOR, which is predominantly facilitated by intracellular overproduction of ROS. Reduced SIRT1 activity was also observed in Tregs from the patients with AAV, but direct activation of SIRT1 ultimately resulted in reduced FoxP3 expression. Additionally, RVL was significantly effective in promoting a reduction in ROS expression with dephosphorylation of mTOR in Tregs, thereby contributing to the increased FoxP3 expression and functional activity. This study suggests that ROS play a pivotal role in inducing plasticity and impaired functional activity of Tregs in AAV. RVL could be useful as an assisting therapy unless conventional immunosuppressive treatment is sufficient to restore Treg stability. However, their

imbalanced homeostatic changes, including higher effector cytokines, lower FoxP3 expression and functional activity than in the HC, remained even after RVL treatment, suggesting that more divergent mechanisms are involved in the imbalance of Treg homeostasis. It is necessary to investigate more precise signaling mechanisms underlying the instability of Tregs in AAV.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shinshu University (approval number: 614). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors made the design of this study, developed the structure and argument for this study. YSh, DK, TI, RT, and SN recruited blood samples and clinical data. YSh performed laboratory investigations, and analyzed obtained data. YSh prepared the draft of this manuscript. YSh and YSe contributed to revise the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.789740/full#supplementary-material

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Dissecting the Landscape of Activated CMV-Stimulated CD4+ T Cells in Humans by Linking Single-Cell RNA-Seq With T-Cell Receptor Sequencing

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CD4+ T cells are crucial in cytomegalovirus (CMV) infection, but their role in infection remains unclear. The heterogeneity and potential functions of CMVpp65-reactivated CD4+ T cell subsets isolated from human peripheral blood, as well as their potential interactions, were analyzed by single-cell RNA-seq and T cell receptor (TCR) sequencing. Tregs comprised the largest population of these reactivated cells, and analysis of Treg gene expression showed transcripts associated with both inflammatory and inhibitory functions. The detailed phenotypes of CMV-reactivated CD4+ cytotoxic T1 (CD4+ CTL1), CD4+ cytotoxic T2 (CD4+ CTL2), and recently activated CD4+ T (Tra) cells were analyzed in single cells. Assessment of the TCR repertoire of CMV-reactivated CD4+ T cells confirmed the clonal expansion of stimulated CD4+ CTL1 and CD4+ CTL2 cells, which share a large number of TCR repertoires. This study provides clues for resolving the functions of CD4+ T cell subsets and their interactions during CMV infection. The specific cell groups defined in this study can provide resources for understanding T cell responses to CMV infection.

Keywords: CMV pp65, single-cell mRNA-seq, paired TCR-seq, CD4+ T cells, CD4+ CTL, Treg

INTRODUCTION

Infections with cytomegaloviruses (CMV) and human herpesvirus 5 (HHV-5) are endemic in humans. Most immunocompetent CMV hosts show few or no clinical symptoms in response to primary infection or during persistent infection. Although CMV infection is asymptomatic, the virus hijacks the resources of the host immune system throughout the latter's lifespan by remaining latent and occasionally reactivating. Over time, CMV-responsive T-cells constitute an average of 10% of the entire T-cell repertoire of the host (1), having deleterious effects on immune senescence and health outcomes in the elderly (2). In addition, CMV infection can have devastating

consequences in immunocompromised populations, including fetuses and patients undergoing transplantation.

Reconstruction of CMV-specific T cells has emerged as an effective method of reducing CMV infection and reactivation in immunocompromised individuals. Data from patients who have undergone hematopoietic stem cell transplantation (HSCT) have shown that recovery from CMV-induced diseases correlates with the reconstruction of CMV-specific CD4+ and CD8+ T-cell pools (3–5), with the recovery of CD4+ T cells regarded as a prerequisite (6). CMV-specific CD4+ T cells are thought to stimulate the expansion of CMV-specific CD8+ T cells, resulting in a more effective clearance of virus from serum than treatment with CD8+ T cells alone (7). Furthermore, infusion of CD4+ T cells into immunocompromised mice was found to effectively repress CMV reactivation, further suggesting a pivotal role of CD4+ T cells in anti-CMV immunity. However, CD4+ T cells are heterogeneous, and their composition, function, and interaction in anti-CMV immunity remain unclear, precluding adoptive immune therapy in CMV-infected individuals.

Studies evaluating the roles of CMV-specific CD4+ T cell subsets in anti-CMV immunity have revealed that CD4+ cytolytic T cells (CD4+ CTL), regulatory T cells (Tregs), and CD4+ memory T cells are involved in immune responses to CMV infection in humans, nonhuman primates, and rodents. CD4+ CTLs were first identified in chronic viral infections, such as with lymphocytic choriomeningitis virus (LCMV), hepatitis B virus (HBV), and CMV. These cells show strong antiviral effects in anti-CMV immunity through their helper functions and induction of cytotoxicity. CD4+ CTLs manifest helper functions through their expression of cytokines and chemokines, such as IFN-γ and TNF-α (8), which promote the activation of CD8+ T cells; recruit innate immune cells, including natural killer (NK) cells and monocytes, to inflammatory sites, and directly inhibit virus replication (9). CD4+ CTLs manifest cytotoxicity through the Fas/FasL pathway, mediating the death of infected B cells presenting viral epitopes with major histocompatibility complex class II (MHC-II) molecules (10, 11). CD4+ CTL also manifest cytotoxicity through the perforin–granzyme pathway (12), based on the CTL recognition of target cells in an MHC-II-dependent manner (13), when MHC-II is upregulated in epithelial cells following CMV infection. Despite advances in understanding the functions of CD4+ CTLs in CMV infection, the derivation of these cells remains unclear. Based on findings in other infectious diseases, CD4+ CTLs are thought to originate from effector cells (14, 15). Recent evidence from studies on transcriptome factors has suggested that these cells can also directly differentiate from activated naïve cells (16–18).

The functions of Treg cells during CMV infection are also unclear. *Ex vivo* stimulation of human Treg cells from CMV-seropositive individuals with CMV was shown to attenuate the proliferation of autologous CD8+ T cells and, to a lesser extent, other subsets of CD4+ T cells through the PD-1 pathway (19). However, CMV reactivation following HSCT did not correlate with the numerical reconstruction of CD4+CD25highCD127- Tregs, and conventional T cells in these patients expressed high levels of the proliferation marker Ki67 indicating that their activation and proliferation were not obstructed by Tregs (20). Selectively deleting Tregs in animal models is a classical method to verify Treg function in infectious situations (21) and has been used to evaluate the negative regulatory function of Tregs in some antiviral immunities. However, deleting Tregs could not determine their function in CMV infection. In mice, the deletion of Treg cells decreased murine cytomegalovirus (MCMV) reactivation in the spleen but enhanced its activation in the salivary glands (22).

CD4+ T cells perform many essential functions, including stimulating B cells to mature and secrete antibodies and supporting cytotoxic CD8+ T cells and phagocytes to mount rapid and effective protection against infections (1). Despite their importance, technical limitations have often prevented the comprehensive analysis of CD4+ T cells. T-cell receptor (TCR) sequences are highly diverse, with an estimated tens of millions of unique TCR-expressing T-cell clones largely unique to individuals (23, 24), limiting the ability to directly compare the abundances of T-cell clones across multiple samples. Antigen-specific T cells can be isolated using peptide-MHC (pMHC) multimers (2), and this method has been used in the parallel detection of T cells on a large scale (3–7). This method, however, depends on advance knowledge of the relevant human leukocyte antigen (HLA) molecules and antigenic epitopes, which in most cases cannot be efficiently predicted (8). In addition, the process involved in generating pMHC multimers is complicated, and few usable pMHC II multimers are available for CD4+ T cells. Due to the variety of HLA alleles (11) and the complexity of many antigen genomes, it is difficult to thoroughly analyze antigen-specific T cells with limited numbers of pMHC multimers. Although the enzyme-linked immune absorbent spot (ELISpot) can also be used to analyze antigen-specific T cells, this method is limited to detecting a single/or a limited panel of cytokine(s) and is therefore not sufficiently comprehensive to analyze different T cell subtypes that are involved in the protection against pathogen infection.

These challenges may be overcome by enriching for T cells specific for CMVpp65 through the expression of the T cell activation marker CD154 induced by stimulation *in vitro*, combined with single-cell mRNA and paired VDJ sequencing to dissect the CD4+ T cell responses (25). This method of isolating CMV-specific CD4+ T cells has several advantages, in that it is HLA-independent, can capture activated CD4+ T cells of different phenotypes, and is useful for high-throughput analysis. Comprehensive analysis of CMV-reactivated CD4+ T cells showed that a large proportion of these cells were CMV-reactivated Treg cells, with a Th1 phenotype, as shown by expression of IFNG and TNF, enhanced migration ability, and multiple inhibitory functions. In addition, this study found that both CD4+ CTL1 and CD4+ CTL2 have polyfunctional phenotypes, experienced clonal expansion, and had a large overlap in TCR repertoire. Furthermore, a group of recently activated CD4+ T cells (CD4+ Tra) cells were found to express cytolytic factor. These findings showed that CMV-reactivated

CD4+ T cells were heterogeneous, consisted of a balance between CMV-specific Treg and effector T cells, and suggested that the composition of CD4+ T cells may be critical for adoptive T cell therapy in patients infected with CMV.

RESULTS

CMV pp65-Specific CD4+ T Cells Have Typical Antiviral Profiles

Circulating antigen-specific T cells are rare in peripheral blood during the latent stage of CMV infection, representing 0.5% to 4% of the CD8+ T-cell pool and 0.05% to 1.6% of the CD4+T cell pool (26). To isolate CMV-specific CD4+ T cells, peripheral blood mononuclear cells (PBMCs) were cultured in the presence or absence of CMV-pp65 peptides for 24 h (25, 27–29). CMV-reactivated CD4+ T cells from three donors were sorted and pooled together for single-cell mRNA-seq and paired VDJ-seq using the 10 × Chromium platform. Single control cells were acquired from each donor by lymphocyte and monocyte sorting with forward scatter and side scatter (FSC/SSC) parameters; the sorted cells were also mixed and subjected to single-cell sequencing (**Figure 1A**). Flow cytometry analysis (**Supplemental Figure 1A**) showed that the expression of CD154 was much higher in CMV-stimulated than in control CD4+ T cells (**Supplemental Table 1**).

After stringent quality control and filtering using multiple criteria, RNA-seq data were obtained from 2,847 and 6,493 single cells from the CMV and control libraries, respectively. These analyses detected a mean of 3,041 genes per CMV infected cell and 1,947 genes per control cell. Productive VDJ sequences were obtained for 1,271 CMV cells and 3,557 control cells. The cells of the three donors from the CMV-infected and control cells were subsequently integrated for further analysis. The unsupervised clustering of all cells in the integrated data resulted in 15 distinct clusters: CD8+ T, γδT, B, NK, mucosal-associated invariant T (MAIT), monocytes, and nine clusters of CD4+ T cells (**Figures 1B–D**). We first showed CD4+ T cells as one cluster to analyze their shared characters and to be able to make comparison with previous studies.

To reveal the potential function of CMV-stimulated CD4+ T cells, CMV and control CD4+ T cells with mRNA and/or productive VDJ data (CMV: 1,200 cells, control: 1,911 cells) were selected for further analysis. Both mRNA and VDJ information was available for 974 cells in the CMV and 1,648 in the control group (**Supplemental Table 2**). Genes differentially expressed by these CMV and control CD4+ T cells were analyzed. CMV CD4+ T cells showed a typical T cell activation profile, including increased expression of *IL2RA*, *TNFRSF4*(*OX40*), *MIR155HG*, *TNFRSF18*, *CD40LG*, and *LGALS1* and decreased expression of *IL7R* and *SELL*. These cells also express genes encoding the inflammatory cytokines *IFNG* and *TNF* (30, 31), the T-bet-independent IFN-γ production inducer *BHLHE40* (32), the pro-inflammatory chemokine *CCL4*, and the cytotoxic molecules *LTA* and *GZMB* (**Figure 1E**). These results suggest that CMV CD4+ T cells consist of several groups of activated multiple-cytokine-producing antiviral cells. These results were further confirmed by Gene Ontology (GO) analysis, which showed that differentially expressed genes (DEGs) were significantly enriched in pathways such as T cell activation and cellular response to tumor necrosis factors (**Figure 1F**). Consistent with previous reports using CD154 as a marker for antigen-specific CD4+ T cells (25), the cells obtained here with the same strategy exhibited a typical activated anti-viral response.

Polyfunctionality Profiles of CMV pp65-Specific CD4+ T Cell Subsets

To date, nine CD4+T cell subtypes have been described (**Figure 2B**), based on markers from our previous study (33) and the Human Cell Atlas (34, 35). Control CD4+ T cells consisted of four clusters: naïve CD4+ T cells/CD4+ central memory like T (Tcm-like) cells expressing *CCR7*, *SELL*, and *TCF7*; CD4+ cytotoxic T2 cells (CD4+ CTL2) expressing *GZMB*, *NKG7*, and *PRF1*; and a Treg cluster expressing Foxp3 and IL2RA. CMV-stimulated CD4+ T cells consisted of five clusters: recently activated CD4+ T (Tra) cells/CD4+ Tcm-like cells expressing CD154 and naïve markers (*CCR7*, *SELL*, and *TCF7*); two cytotoxic T cell clusters (CD4+ CTL1 and CD4+ CTL2) expressing *GZMB*, *NKG7*, and *PRF1* and distinguished by different expressions of chemokines (CD4+ CTL1 highly expressed *CCL5*, CD4+ CTL1 highly expressed *CCL3* and *CCL4*); a Treg cluster expressing *Foxp3* and *IL2RA* (**Table 1** and **Figures 2A–C**); and CD4+ central memory-like T cells and CD4+ naïve T cells which were further discriminated by GSEA analysis, as DEGs between CD4+ naïve T and CD4+ Tcm-like cells significantly enriched in the gene sets such as "GSE11057 NAÏVE VS MEMORY CD4 TCELL DN" and "GSE11057 NAÏVE VS CENT MEMORY CD4 TCELL DN" (**Supplemental Table 3**). The proportions of each subtype are shown in **Figure 2D**. The ratio of naïve to memory control CD4+ T cells was consistent with previous fluorescence-activated cell sorting (FACS) data (36). To attribute cells to their corresponding donor, PBMCs from the three donors were subject to bulk RNA-seq for subsequent single-nucleotide polymorphism (SNP) identification, and the identity of each cell was determined based on these natural genetic variations (37). Cells from donor 1 and donor 2 were generally similar (**Supplemental Figures 2A–C**). Few cells were obtained from donor 3, with this donor accounting for 1.58% of the total CD4+T cells from the three donors. These results showed that CMV-stimulated CD4+ T cells were highly enriched in Treg cells and CD4+ CTLs.

To investigate the transcriptome features of the five CMV-stimulated CD4+ T cell subsets, CD4+ T cells from the CMV dataset (1,200 cells) were selected for further analysis. The five CD4+ T cell subsets were compared with each other using the *FindAllMarkers* function, with the resulting DEGs shown in **Supplemental Table 4**. The top 10 DEGs (sorted by the logFoldChange parameter) were found to differ from each other, indicating that these subsets may have distinct phenotypes (**Figure 2E**). The phenotype of each subset was

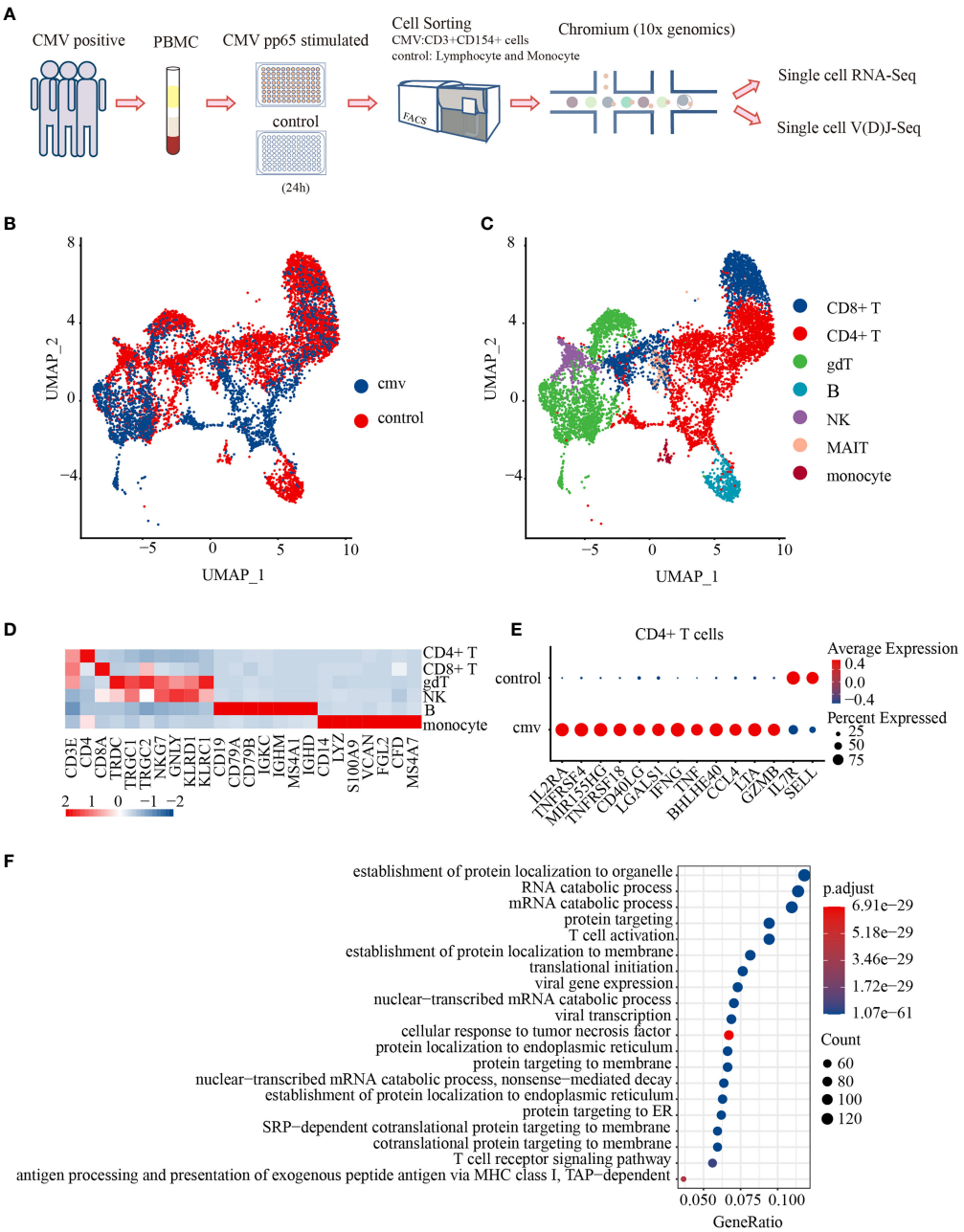

FIGURE 1 | Characterization of the antiviral profiles of CMV pp65-specific CD4+ T cells. **(A)** Experimental workflow for single-cell analysis of CD4+ T cells from PBMC of three donors. Cells stimulated *in vitro* with CMV pp65 were cultured and sorted, with reactivated CMV-stimulated T cells gated for CD3+CD154+, and control monocytes and lymphocytes gated for FSC-SSC, followed by 5′ single-cell RNA and paired T-cell receptor sequencing. UMAP embeddings of merged scRNA-seq profiles from control and CMV-stimulated immune cells were plotted and colored by **(B)** sample and **(C)** cell cluster. **(D)** Heat map of scaled mean gene expression of the major canonical markers (columns) detected in different cell types in merged CMV and control cells (rows). **(E)** Dot plot of differentially expressed genes (DEGs), showing both the levels of expression and the percentages of CD4+ T cells in CMV and control samples. **(F)** Gene Ontology (GO) analysis of DEGs in CMV and control CD4+ T cell samples. The Top 20 enriched GO terms are ordered on the y-axis. The x-axis represents the gene percentage in enriched GO terms. The sizes of the dots represent the number of genes included in each GO term. The color gradient of dots represents the adjusted p-values of each enriched GO term.

therefore analyzed based on the top 10 DEGs and feature genes previously identified in these subsets.

To understand the phenotype and role of Treg cells during CMV infection, their gene expression profiles were analyzed. These cells are *FOXP3+IL2RA+TNFRSF4+*, as well as expressing proinflammatory factors such as *IFNG* and *TNF*. When compared with the four other CMV-stimulated CD4+ T subsets (i.e., CD4+ Tra cells, CD4+ Tcm-like cells, CD4+

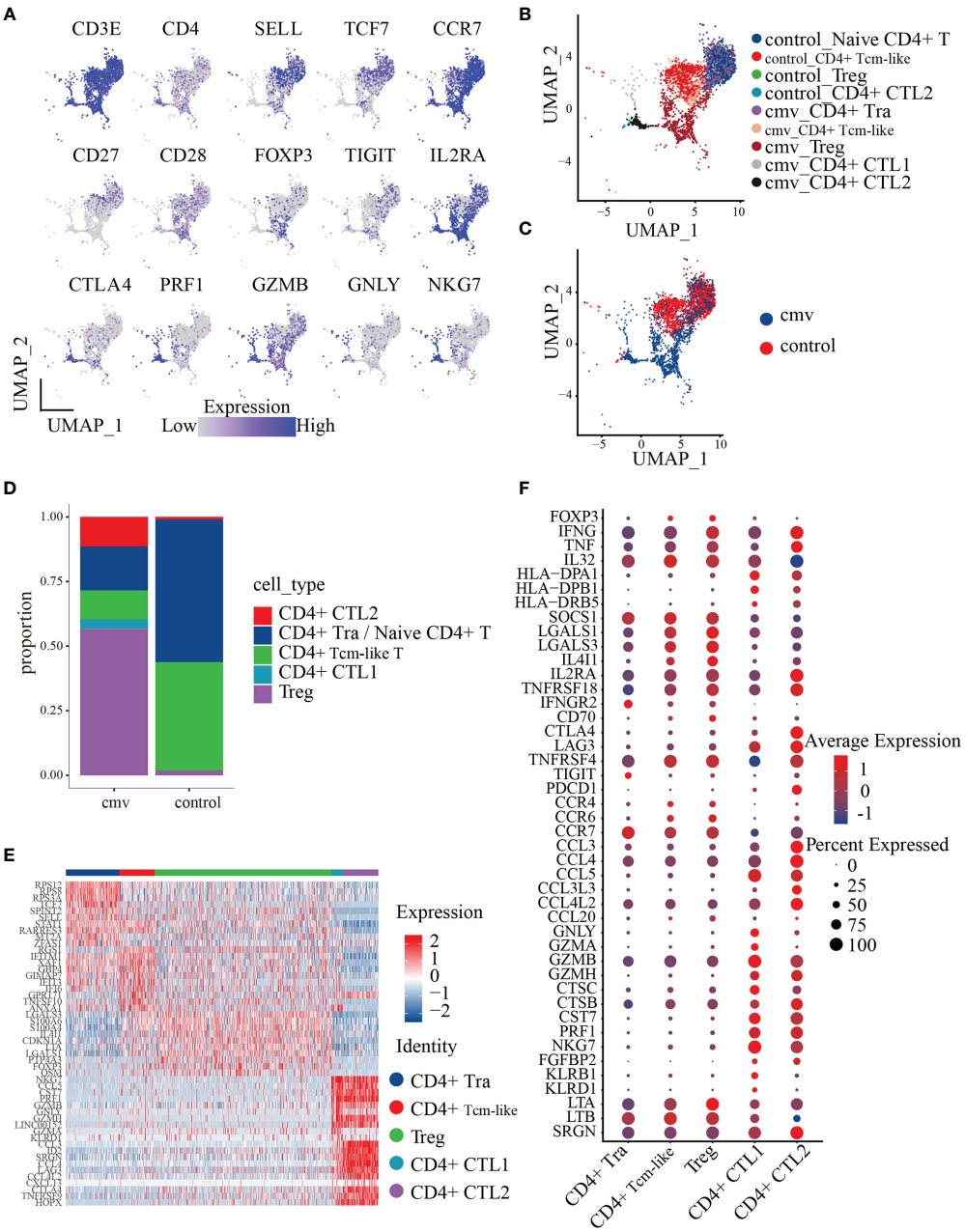

FIGURE 2 | Polyfunctionality profiles of CMV pp65-stimulated CD4+ T cell subsets. **(A)** UMAP projections for the merged CD4+ T cells colored by expression of the naïve CD4+ T/Tcm-like cell markers *CD3E*, *CD4*, *SELL*, *TCF7*, *CCR7*, *CD27*, and *CD28*; Treg markers *FOXP3*, *IL2RA*, and *TIGIT*; the cytotoxicity markers *GZMB*, *NKG7*, and *PRF1*. Relative expression was normalized across CMV and control datasets. **(B, C)** UMAP embeddings of merged scRNA-seq profiles from control and stimulated (CMV) CD4+ T cells plotted and colored by cell cluster **(B)** and sample **(C)**. Subpopulations of CD4+ T cells colored in **(B)** were identified by the canonical markers described in **Table 2**. **(D)** Distribution of the abundance of the subsets of CMV and control CD4+ T cells. **(E)** Heat map of the five subsets of CMV CD4+ T cell cells with the Top10 DEGs between each pair. **(F)** Dot plot of highly featured genes expressed in the five CD4+ T cell subsets in CMV.

CTL1, and CD4+ CTL2 cells), the Treg cells showed a significantly higher expression of the stable marker *SOCS1*, the cytotoxicity-related molecule *LTA*, and a series of proteins encoded by genes related to inhibition, such as *LGALS1* (38), *LGALS3* (39), and *IL4I1* (40) and the costimulatory molecule *CD70* (adjusted $p < 0.01$ each) (**Figure 2F**). The expression by Tregs of the chemokine receptors *CCR4*, *CCR6*, and *CCR7* indicate their chemotaxis toward *CCL3* and *CCL5*, the latter of which is highly expressed by CD4+ CTL1 and CD4+ CTL2 cells, and the homing to secondary lymphoid organs. Moreover, the high level of expression of *CCL20*, which encodes a chemokine that binds to CCR6 in Tregs, suggests

TABLE 1 | Cell type markers.

Cell type	Markers
Naïve CD4+ T/Tcm-like	CD3E+, CD4+, SELL+, CD27+, TCF7+, CCR7+
CD8+ T	CD3E+, CD8A+, CD8B+, CD4-
γδT	CD3E+CD4-CD8B-CD8aa+/-, TRDC+, TRGC1+, TRGC2+
Treg	CD3E+, CD4+, FOXP3+, IL2RA+
Recently activated CD4+ T	CD3E+, CD4+, SELL+, TCF7+, CCR7+, CD154+
B	CD19+, CD79A+, CD79B+, MS4A1+, IGKC+, IGHM+
NK	CD3E-, NKG7, GNLY, NKG7, KLRD1, KLRC1
CD4+ CTL1	CD3E+, CD4+, CD27-, CD28-, GZMB+, NKG7+, PRF1+, CCL3+, CCL4+
CD4+ CTL2	CD3E+, CD4+, CD27-, CD28-, GZMB+, NKG7+, CCL5
Monocyte	LYZ+, S100A9+, CD14+, FGL2+, MS4A7+
MAIT	TRAV1-2/TRAJ33, TRAV1-2/TRAJ20, TRAV1-2/TRAJ12

that these cells cluster in a self-sustaining positive feedback loop.

CD4+ CTLs play an important role in chronic antiviral responses and contribute directly to the containment of viral infection. Assessments of the phenotypes and functional mechanisms of the five CD4+ T subsets showed that both CD4+ CTL1 and CD4+ CTL2 expressed high levels of genes encoding cytotoxic molecules, including *GZMB*, *GZMH*, *CTSC*, *CTSB*, *CST7*, *PRF1*, *NKG7*, and *FGFBP2* (**Figure 2F**). The similar levels of expression of these cytotoxic markers in CD4+ CTL1 and CD4+ CTL2 indicate that they may employ the same mechanism of action, the granule exocytosis pathway, to initiate target cell apoptosis. This mechanism involves the regulated release of the contents of cytotoxic granules (e.g., PRF1, GZMB, GZMH, GZMA, CTSC, and GNLY) into the immunological synapses formed between effector and target cells, killing the latter (41). CD4+ CTL1 and CD4+ CTL2 also expressed high amounts of the chemokine CCL5 and the MHCII molecules HLA-DPA1 and HLA-DPB1, indicating that they may attract common targets to inflammatory sites and kill them in an MHC class II-dependent manner (13, 42). Besides, compared with CD4+ CTL2, CD4+ CTL1 expressed higher levels of many other cytotoxic molecules, such as GNLY, GZMA, KLRB1, and KLRD1 (**Figure 2F**), indicating that the functional spectrum of CD4+ CTL1 is wider than that of CD4+ CTL2. When compared with CD4+ CTL1, CD4+ CTL2 expressed higher levels of many genes encoding chemokines (such as *CCL3*, *CCL4*, *CCL3L3*, and *CCL4L2*, and co-stimulators, such as *CTLA4*, *LAG3*, *TNFRSF4*, and *PDCD1*), indicative of a terminal differentiation phenotype. These results suggest that CD4+ CTL2 may originate from CD4+ CTL1 cells, which is further supported by our TCR repertoire analysis.

CD4+ T cells recently activated by exposure to CMV pp65 peptides were found to cluster together with control naïve CD4+ T cells. Sorting of recently activated CD4+ T (Tra) cells by CD154 expression showed that these cells express high levels of genes encoding naïve T cell markers, such as *CCR7*, *TCF7*, and *SELL* (**Figures 2A–C**). To dissect the phenotype of the CD4+ T cells recently activated by CMV, we compared their gene expression with that of control naïve CD4+ T cells. In total, 981 genes were differentially expressed (adjusted $p < 0.05$) upon stimulation with the CMV pp65 peptides (**Figure 3A** and **Supplemental Table 5**). Of these, 121 genes were upregulated in CMV-activated cells and 36 were downregulated, with log2-fold changes > 1. These 121 upregulated genes included a group of genes encoding the cytokines and chemokines (*IFNG*, *TNF*, *LTA*, *MIF*, *IL32*, *CXCL10*, and *CCL4L2*), a group of genes regulating protein synthesis (e.g., *WARS*, *SEC61G*, and *EIF5A*), and a group involved in metabolism (43, 44) (e.g., *GAPDH*, *PKM*, *ENO1*, *TPI1*, and *PGK1*) (**Figure 3B**), findings indicative of cell activation (45). CD4+ Tra cells also expressed higher levels of S100 family genes encoding calcium-binding proteins (e.g., *S100A4*, *S100A10*, and *S100A11*) and cytoskeleton-related proteins (e.g., *ACTG1*, *ACTB*, *TUBB*, *PFN1*, and *MYO1G*), which had been reported increased in response to TCR engagement by antigen (46, 47). In addition, genes encoding many regulatory markers (e.g., *GITR* [*TNFRSF18*], *CISH*, *SOCS1*, and *TIGIT*) and cell apoptosis regulation markers (e.g., *LGALS1*, *FAM162A*, *CFLAR*, *FAS*, and *CDKN1A*) were strongly upregulated to maintain immune balance (48), although their expression levels differed in cells at different stages of differentiation (49, 50). The 36 downregulated genes included *CD127 (IL7R)*, *CD27*, and *SELL*, consistent with previous studies of T cell activation (51). GO analysis of the DEGs in recently activated CMV pp65-stimulated CD4+ T cells and control naïve CD4+ T cells demonstrated the significant enhancement of expression of genes associated with T cell activation, protein targeting, cellular response to tumor necrosis factor, viral gene expression, protein targeting to membrane, and the tumor necrosis factor-mediated signaling pathway (**Figure 3C**). These findings suggested that these phenotypically naïve CMVpp65-stimulated cells are in a state of recent activation.

CMV pp65-Specific CD4+ T Cell Receptor Repertoire Shows a Reduction in Clonal Diversity

The T-cell receptor (TCR) repertoire reflects the antigen specificity of T cells and their antigen experience in effector and memory subsets. Compared with the clonal diversity of the control CD4+ TCR repertoire, the clonal diversity of the CMV pp65-specific CD4 + TCR repertoire was reduced. Clones with the same VDJ (gene) and CDR3 nucleotide (nt) sequence were defined as being of the same clonotype (gene+nt), followed by a comparison of the features of the CD4+ TCR repertoire in CMV-stimulated and control cells. Analysis of the relative abundance of total CMV-stimulated and control CD4+T cells showed that the percentages of unique (i.e., unexpanded) clones in the CMV and control CD4+ T cells were 90.20% and 99.27%, respectively (**Figure 4A**). About 9.8% of the

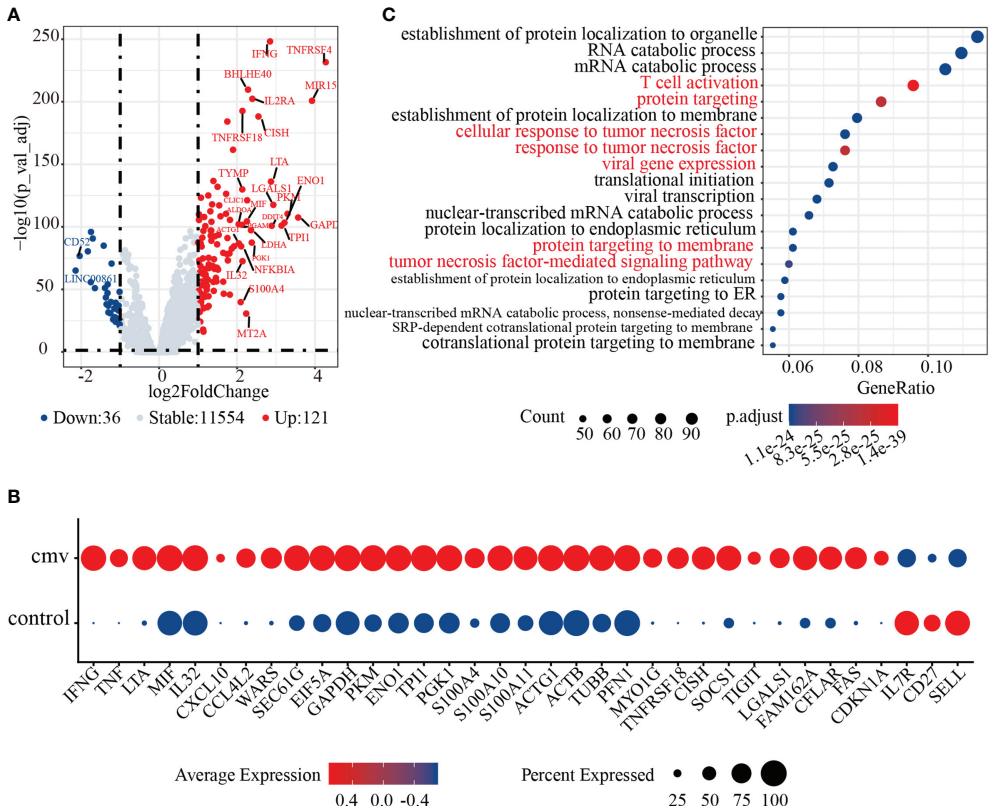

FIGURE 3 | Activation characteristics of CMV pp65 stimulated recently activated CD4+ T (CD4+ Tra) cells. **(A)** Volcano plot showing the relationships between -log10(adjusted p value) (y-axis) and log2(fold change) (x-axis) for genes differentially expressed by CMV CD4+ Tra and control naive CD4+ T cells. Genes with log2 fold changes > 1 and adjusted p values < 0.05 were upregulated in CMV CD4+ Tra cells and highlighted in red, whereas genes with log2 fold changes < -1 and adjusted p values < 0.05 were downregulated in CMV CD4+ Tra cells and highlighted in blue. **(B)** GO analysis of DEGs by CMV CD4+ Tra cells and control naïve CD4+ T cells. The Top 20 enriched GO terms are ordered on the y-axis. The x-axis indicates gene percentages in enriched GO terms. The sizes of the dots represent the number of genes included in each GO term. The color gradient of dots represents the adjusted p-values for each enriched GO term. **(C)** Dot plot of highly featured genes expressed by CMV CD4+ Tra cells and control naïve CD4+ T cells.

CMV-pp65-stimulated CD4+ T cells showed "medium" or "large" expansion (**Figure 4B**), indicating that they had undergone clonal amplification. Measured diversity using Shannon, Inverse Simpson, Chao, and abundance-based coverage estimator (ACE) across samples also showed an overall reduction in clonal diversity in the CMV sample (**Figure 4C**). To identify clones targeting the same antigens among cell subsets, the GLIPH2 algorithm (52) was utilized to cluster clones of CMV and control CD4+ T cells. The TCR convergence was found to be higher for CMV than for control CD4+ T cells (**Supplemental Tables 6** and **7**), with the TCR repertoire convergences being mainly between CD4+ CTL1 and CD4+ CTL2 in CMV. Consistent with the GLIPH2 result, combining VDJ sequences with transcriptome data (**Supplemental Table 8**) showed that the "larger" and "medium" expanded clones were mainly in the CD4+ CTL1 and CD4+ CTL2 subsets (**Figure 4D**).

TCR Repertoire in CMV-Stimulated CD4+ T Cell Subgroups

To determine the dynamic changes in the CMVpp65-specific TCR repertoires of CD4+ T cell subsets, we analyzed the TCR repertoire of the five subgroups of CMV-stimulated CD4+ T cells. Measured TCR diversity using Shannon, Inverse Simpson, Chao, and ACE across these five cell clusters consistently showed reductions in clonal diversity in the order Treg, CD4+ Tra cells, CD4+ Tcm-like cells, CD4+ CTL1, and CD4+ CTL2 (**Figure 5A**). Calculation of the overlap in TCR repertoire among these clusters using overlap coefficient methods showed a large clonal overlap between CD4+ CTL1 and CD4+ CTL2 (**Figure 5B**); the VDJ sequences shared by these are shown in **Supplemental Table 8**. Evaluation of cloning frequency showed that the CD4+ CTL1 and CD4+ CTL2 clones experienced larger or medium expansion, the CD4+ Tcm-like and Treg cell clones experienced small or no expansion, and the CD4+ Tra cell clones experience no expansion (**Figure 5C**). Analysis of the transcriptome similarity of these clusters showed that CD4+ T-cell clones with the same receptor sequence had more similar gene-expression profiles than non-clonally expanded T cells (CD4+ CTL2 vs. CD4+ Tra cells, $p < 2.2e\text{-}16$; CD4+ CTL2 vs. Treg cells, $p < 2.2e\text{-}16$; CD4+ Tra vs. Treg cells, $p < 2.2e\text{-}16$; by paired Wilcoxon test), as shown by comparing the Jaccard similarity coefficients for the 200 most abundant genes chosen

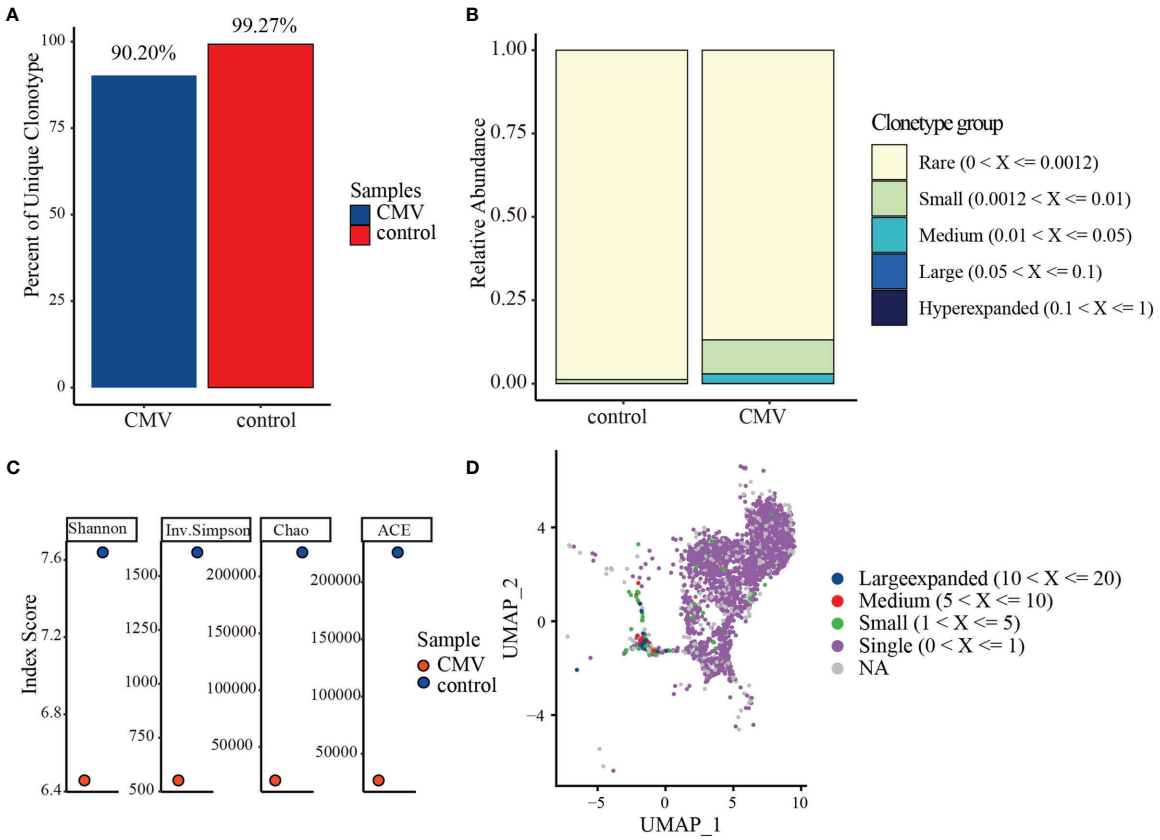

FIGURE 4 | TCR repertoire analysis of CMV and control CD4+ T cells. **(A)** Percentages of unique (i.e., unexpanded) clonotypes of CMV and control CD4+ T cells. **(B)** Relative TCR repertoire abundance of CMV and control CD4+ T cells. **(C)** Diversity measures based on clonotypes by sample type using Shannon, Inverse Simpson, Chao, and abundance-based coverage estimator (ACE) indices. **(D)** Clonotype distributions of CD4+ T cells. Cloning frequencies ≤20 and > 10, ≤10 and >5, ≤5 and >1 were defined as large expanded, medium expanded, and small expanded, respectively.

from each cell type cluster (53) (**Figure 5D**). It is highly possible that CMV-reactivated CD4+ CTL1 and CD4+ CTL2 may be different states of the same group.

DISCUSSION

Although CD4+ T cells have been shown to play a significant role in anti-CMV immunity, previous methods of measuring CD4+ T cell responses have provided only a partial picture of the involvement of CD4+ T cells in immunological responses to CMV. This study presents a comprehensive profile of CMV pp65-specific CD4+ T cell responses. First, it showed that, of these T cell populations, a surprisingly high percentage (56.68%) consisted of Tregs, with the remaining effector cells being predominantly polyfunctional cells with cytotoxic profiles. Second, this study found that CD4+ CTL2 cells are a more differentiated subset of CD4+ CTL1 cells, evidenced in part by their overlapping TCR repertoires. A key advantage of this study was the use of overlapping pp65 peptide stimulation and CD154 as indicators of CD4+ T cell activation, both of which are independent of MHC haplotype. These results enable further characterization of the CMV-specific CD4+ T cell response and can be compared with responses to other viruses.

CD154 is an effective marker when combined with single-cell mRNA sequencing for high-throughput analysis of virus antigen-specific T cells (25, 27–29). Although traditional research methods based on measurement of secreted cytokines, such as IFNG or TNF, and testing of CMV-specific T cells have proven effective (54–56), they are of limited use when combined with sc-mRNA sequencing due to cell damage caused by intracellular staining. The use of peptide-MHC (pMHC) multimers to isolate antigen-specific T cells based on the specific binding of TCR with pMHC has allowed detailed TCR and phenotypic analysis of single cells (57–59). However, the decreased TCR expression in activated T cells can result in the selection of relatively low antigen-specific T cells bound to tetramer (60), This selection of multimer-binding CD4+ T cells may bias understanding of the phenotype of antigen-specific CD4+ T cells (60). The finding that 83.8% of CMV stimulated but only 17.4% of control CD4+ T cells were positive for *CD154* (*CD40LG*)

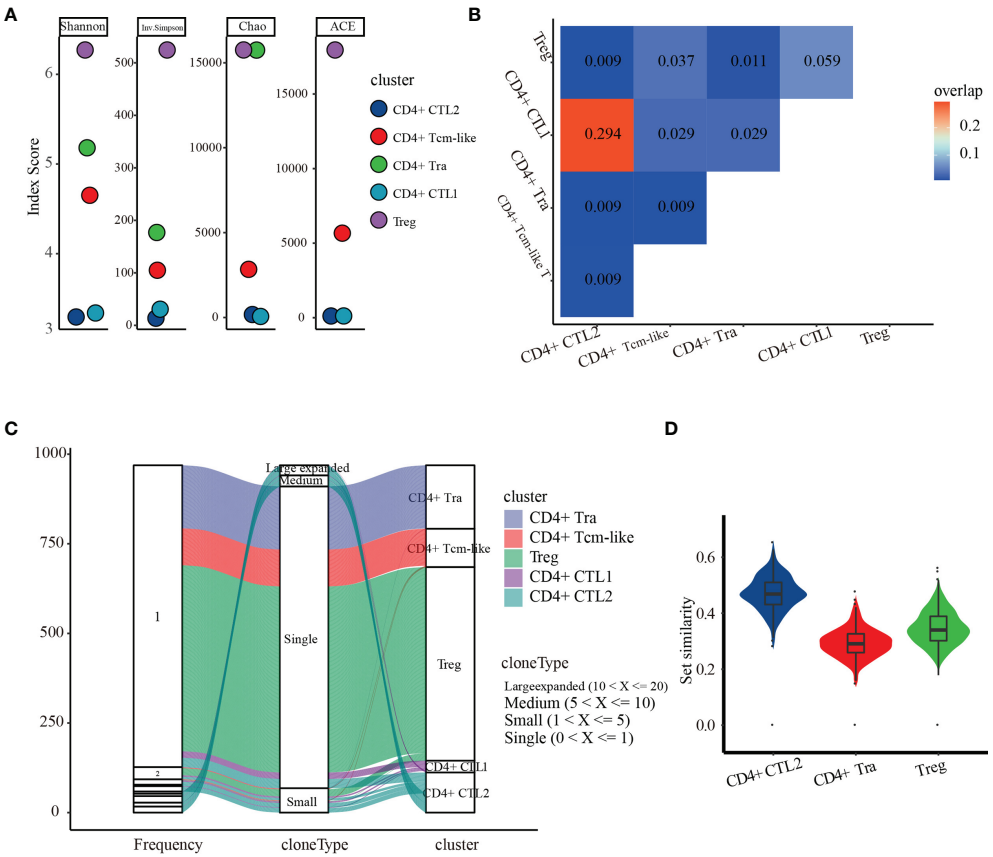

FIGURE 5 | TCR repertoire analysis of the five CMV CD4+ T cell subsets. **(A)** Diversity measures based on clonotypes by cluster using Shannon, Inverse Simpson, Chao, and abundance-based coverage estimator (ACE) indices. **(B)** Clonal overlaps among the five CMV CD4+ T cell subsets. **(C)** Cloning frequency distribution in each subset. **(D)** Transcriptome similarity among CMV CD4+ CTLs, CD4+ Tra cells, and Treg cells.

expression indicates that CD154 is comparable to IFNG and TNF in distinguishing antigen-specific CD4+ T cells.

This study found that the CMV-reactivated Tregs had different inhibitory functions. LAG3 and CTLA4 are classical Treg inhibitory markers, which bind to MHC-II and CD80/CD86, respectively, on other T cells to repress their activation. Perforin/granzyme-induced apoptosis is the main pathway used by cytolytic cells to kill target cells (61, 62), with perforin and granzyme commonly expressed simultaneously. In our study, Tregs were positive for *SRGN*, which encodes a protein involved in maintaining granzyme storage, and highly expressed *GZMB*, but their expression of *PRF1* was limited. These findings suggest that only a few perforin molecules are sufficient to facilitate the entrance of granzyme into target cells, or that granzyme B can induce cell death in a perforin-independent manner (63), by mediating the cleavage of the extracellular matrix to reduce the adhesion of immune cells, inducing their death. These cells also expressed *LGALS1* and *LGALS3*, encoding Gal-1 and Gal-3, respectively, which may also participate in Treg immunosuppressive activity (64). Disruption of Gal-1 was found to attenuate the immunoexpressing effect of Treg cells (65), and Gal-1 from Tregs was observed to induce the dysfunction of effector T cells and modulate their transient calcium influx (66). This regulatory mechanism is not limited to Gal-1 but is also employed by Gal-3 in Tregs (67). Interestingly, this study showed that Tregs expressed CD70, a marker, to our knowledge, commonly expressed on antigen-presenting cells and activated T cells as part of the CD27-CD70 pathway that provides a costimulatory signal. In T cells, CD70 was shown to induce caspase-dependent apoptosis. Although the mechanism by which Tregs exert inhibitory activity may be similar (68), additional studies are needed to determine the function of CD70 in Tregs. Taken together, these findings show that, during CMV infection, the inhibitory activity of Treg cells is not only maintained but reinforced by enhancing cell migration.

The populations of Treg/induced Tregs (iTregs) have been reported to increase during CMV/MCMV latent infection both in humans and in mice (22, 69–72). However, it is not clear whether these increases are due to the expansion of a small population of circulating Forxp3+ nTregs or due to peripheral conversion of antigen-specific CD4+T cells into iTregs. Most of the Tregs in the present study were probably induced from conventional T cells by TGFβ, which is secreted by all CD4 T

subsets and maybe by other cell types in PBMC cultures. Moreover, Tregs were found to inhibit immune responses in the spleen but promote virus control in the salivary glands, suggesting that the effects of Tregs are dependent on their location. It is more likely that, in the presence of functional CD4 CTL, the immune system would favor iTregs over newly activated T cells, especially in the peripheral blood, where inflammation can be more harmful than in a relatively restricted tissue.

During acute viral infection, CD4 + T cells assist in the activation of CD8 + T and B cells to clear the virus. During chronic infection, including infections with HCMV, MCMV, herpes simplex virus, varicella zoster virus, murine gammaherpesvirus 68, and Epstein–Barr virus, CD4 + T cells play a direct antiviral role, inhibiting virus lysis and replication. This can result in the establishment of virus latency and prevent disease or death in the host (73–75). It is unclear what mechanisms contribute to the establishment of cytotoxic CD4T in chronic infection. In our study, we found populations of activated CD4 CTLs among large numbers of Tregs. CD4 CTLs induced by latent viruses are independent of co-stimulation, resistant to apoptosis, and less susceptible to suppression by regulatory T cells (Tregs) during repeated antigenic stimulation (76). Interestingly, the number and proportion of CD4 CTL cells expressing immune regulating genes, such as *CTLA-4*, *LAG3*, *IL-2RA*, and *PDCD1*, were at least comparable to, if not greater, than the number and proportion Treg cells. Fewer less resources are therefore available for the activation of other conventional CD4+ T cells. In addition, both CD4 CTLs and Treg cells express IFN-γ and TNF-α, which can promote innate immune responses. Although this study did not determine whether IFN-γ and IFN-expressing Treg cells have enhanced or dampened function, it is likely that the combination of CD4 CTLs and Tregs will result in CD4 CTL dominant immune responses accompanied by increased innate immune responses.

Less is known about bystander activation of CD4+ T cells than of CD8+ T cells, but unrelated memory CD4+ T cells were shown to be activated after repeat tetanus vaccination *via* bystander activation (77), and multiple cytokines sharing a common receptor gamma chain were found to induce CD154/CD40 ligand expression by human CD4+ T lymphocytes *via* a cyclosporin A-resistant pathway (78). We found that CD4+ Tcm-like cells, which exist in an environment containing IFN-γ and IL2, are susceptible to activation by these cytokines. We also found, however, that CMV CD4+ Tcm-like cells showed small clonal expansion, making it difficult to determine whether these CD4+ Tcm-like cells are CMV pp65 antigen-specific.

The present study provides useful information for the characterization of CMV-specific CD4 T cell responses and for comparisons with other virus-specific responses. The method we used to analyze CMV-reactivated CD4+ T cells may be extended to other conditions, such as autoimmune diseases and cancers. Our findings may offer insights into the persistence of CMV and levels of immunopathology. In addition, the detailed information provided in this study, such as cell function and cell interactions, may provide a more nuanced view of CMV-related diseases and allow better design of anti-viral therapies.

METHODS AND MATERIALS

PBMC Preparation

We obtained peripheral blood from three CMV IgG-positive, healthy donors through a research protocol proved by the Beijing Genomics Institution-Shenzhen (BGI-Shenzhen) Institutional Review Board (IRB). PBMCs were immediately isolated from blood collected with an EDTA blood collection tube by density centrifuge method with Histopaque-1077 (Sigma, Cat. 10771) within 2 h, resuspended in 4°C cryopreservation medium consisting of 90% fetal bovine serum (FBS, HyClone, Cat. sh30084.03) and 10% dimethyl sulfoxide (DMSO, Sigma, Cat. D4540), and then placed in Mr. Frosty (Thermo Scientific) in -80°C container. Samples were then moved to liquid nitrogen for long-time storage.

Additionally, 2 ml peripheral blood from each donor was collected using a blood collection tube without any additive, placed at room temperature for 30 min, and centrifuged for 10 min at 2,000g. Then, plasma was collected and heat-shocked for 30 min at 55°C.

PBMC Stimulation

Frozen PBMC from liquid nitrogen were immediately thawed in 37°C water and resuspended in complete medium (RPMI 1640 medium, 10% NEAA, and 2% autologous plasma; RPMI 1640 and NEAA were purchased from Thermo Fisher with Cat. 72400120 and Cat. 11140050) to a final density of $1*10^7$ per milliliter (ml). We moved 150 µl of cell suspension with three repetitions to each well in the 96-well U-plate (Falcon) and incubated them at 37°C for 2 h. Then, 75 µl culture supernatant in each well was replaced by 75 µl stimulation medium and gently mixed. Cells were cultured in an incubator with 5% CO_2 at 37°C for 24 h.

The stimulation medium included RPMI 1640 medium (without serum), anti-CD28 (2 µg/ml, Clone G28.5, GeneTex, Cat. GTX14148), and anti-CD40 (2 µg/ml, Clone HB14, Miltenyi, Cat. 130-094-133) with/without CMV peptide (1.2 nmol/ml per peptide). To preserve the surface expression of CD154 on activated T cells, we used anti-CD40 to inhibit the interaction of surface CD154 with its counterpart CD40 as described in the previous study (25). We stimulated PBMCs from three CMV-seropositive donors *in vitro* with CMVpp65 peptides in the presence of anti-CD40 monoclonal antibody, negative control cultured without CMVpp65, and positive control with anti-CD3 and anti-CD28. The CMV pp65 peptide was purchased from Miltenyi (Cat. 130-093-438) and diluted in sterile water.

Enrichment of CMV pp65-Specific T Cells

Cells were collected and washed with FACS washing buffer (DPBS, 2% FBS, and 1 mM EDTA) for once and resuspended in staining buffer (FACS washing buffer with 10% human

plasma and 1% BSA) containing antibodies against CD3, CD4, CD154, and CD69 (**Table 2**). After being incubated on ice for 40 min, cells were washed with FACS washing buffer twice and resuspended in 100 µl washing buffer. The stained cells were analyzed and sorted by a BD FACS Aria II cell sorter (BD Biosciences). For cells stimulated with the CMV peptide, CD3+CD154+ cells were sorted as CMV-specific T cells. For unstimulating cells, monocytes and lymphocytes gated according to the plot of FSC-SSC were sorted respectively and re-mixed as a control. The gating schedule for cell sorting was recorded by BD Aria II, and FACS data were analyzed with FlowJo v10.0.7.

Droplet Generation, 10× RNA-Seq, and TCR-Seq Library Preparation and Sequencing

After being counted with C-Chip (inCYTO), CMV-reactivated cells and control cells from all three individuals were mixed separately and diluted with PBS to a final concentration of ~800 cells/µl, and about 20,000 cells per reaction were loaded onto a Chromium Single Cell Chip (10x Genomics). The libraries for RNA-seq and TCR-seq were prepared using the Chromium Single Cell 5′ Library & Gel Bead Kit v2 and Chromium Single Cell V(D)J Human T Cell Enrichment Kit (10x Genomics) following the manufactory's protocol. Sequences within these libraries were ligated with BGIseq adapters, and then CMV and control libraries were loaded onto the sequencing chip. The RNA-seq libraries were sequenced with an 8-base index read, a 26-base read 1 containing cell-identifying barcodes and unique molecular identifiers (UMIs), and a 100-base read 2 containing transcript sequences on BGIseq500; TCR-seq were sequenced with an 8-base index read, a 150-base read 1 containing cell-identifying barcodes, UMIs and insert starting from the V-gene region, and a 150-base read 2 containing an insert from the C-gene region. The raw data after sequencing were about 10 + 35 Gb per library for RNA-seq and 35 + 35 Gb for TCR-seq.

Preprocessing Single-Cell RNA-Seq Data

Raw data were split according to sample barcodes into CMV-stimulated (ST) and unstimulated library (CON) and then were filtered, blasted, aligned, and qualified by Cellranger v2.2.0 with reference of refdata-cellranger-GRCh38-1.2.0 for RNA-seq data and Cellranger v3.0.0 with refdata-cellranger-vdj-GRCh38-alts-ensembl-2.0.0 for TCR-seq data. Other parameters were set as default in the software.

Data Integrating and Cell Clustering

The R package Seurat (79) 3.1.5 was used to integrate and analyze datasets from CMV and control. The merged expression matrix was firstly filtered following the Seurat recommendation (80, 81) and a total of 8,671 cells with unique UMI was obtained. Unsupervised clustering was conducted with Seurat with the parameter res = 0.5.

Differential Expression Gene Analysis

Differential expression gene (DEG) analysis was conducted by the function *FindMarkers* provided by *Seurat*. To characterize the features of CMV-specific CD4+ T cell response, we used a stricter standard to filter out DEGs between CMV and control CD4+ T cells according to the following standard: for upregulation genes in CMV, adjusted p-value < 0.05, log fold change >1, percentage of cells expressing the gene in the CMV sample (pct.1) >0.8, percentage of cells expressing the gene in control (pct.2) < 0.2; for downregulation genes in CMV, adjusted p-value < 0.05, logFC >1, pct.1 <0.2, pct.2 >0.8.

Quality Control Metrics and Filtering

CellRanger v2.2.0 software with default settings was used to process the raw FASTQ files, align the sequencing reads to the GRCh38 transcriptome, and generate a filtered UMI expression profile for each droplet.

Identifying the Sample Identity of Each Droplet

The transcriptome of each donor's PBMCs was sequenced on the BGI-SEQ500 platform with sequencing type SE200. Raw data with 10 G per sample were obtained. The best-practice workflows recommended by the Genome Analysis Toolkit (GATK) (https://gatk.broadinstitute.org/hc/en-us/articles/360035531192-RNAseq-short-variant-discovery-SNPs-Indels-) were followed to identify single-nucleotide polymorphisms (SNPs) and create VCF files containing the genotype (GT) to assign each barcode to a specific sample. The VCF file and BAM files produced by CellRanger2 were passed to the demuxlet software to deconvolute sample identity (37). The optimal likelihood for the identity of each sample was assigned to the corresponding donor, with each "possible" or "ambiguous" droplet regarded as unclear.

GO Analysis

To annotate the potential functions of the DEGs of each CD4+ T cell cluster, GO enrichment analysis was performed using the clusterProfiler R package, version 3.14.3 (82), with the

TABLE 2 | FACS antibodies.

Antigen	Clone	Fluorophore	Supplier	Dilution
CD3	SK7	FITC	BioLegend	1:100
CD4	RPAT4	PerCP-Cy5.5	eBioscience	1:200
CD154	TRAP-1	PE	BD	1:50
CD69	FN50	BV421	BioLegend	1:50

differentially expressed feature genes identified by Seurat. The top 20 enriched pathways, ranked by normalized enrichment score, with Franklin Delano Roosevelt (FDR) q-value ≤0. 05 were chosen and visualized.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA, http://www.broad.mit.edu/gsea) was performed with default sets to determine the cell type of cluster 3. The gene set collection used for GSEA was c7.all.v7.1.symbols.gmt (ftp.broadinstitute.org://pub/gsea/gene_sets/c7.all.v7.1.symbols.gmt).

TCR Analysis

TCR analyses were performed with the R package scRepertoire and Gliph2. Overlap coefficients were calculated using the intersection of clonotypes divided by the length of the smallest component.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been deposited into the CNGB Sequence Archive of CNGBdb with accession number CNP0001262 (https://db.cngb.org/search/sample/?q=CNP0001262).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethical clearance from the institutional review board of BGI. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LT and XL designed this project. MHL and SYW performed experiments together. MHL conduct data analysis. MHL, LT, SYW, KG, LW interpreted the data and drafted the manuscript. LT, MHL, BL, XJZ, MNW revised the manuscript. YL modified the syntax. LT, XL, and BL provided direction. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.779961/full#supplementary-material

Supplementary Figure 1 | Flow cytometry analysis of cells from the three CMV seropositive donors stimulated with anti-CD3 and anti-CD28 antibodies, stimulated with CMVpp65 peptides, and unstimulated (control). FACS data are missing for unstimulated cells from donor #1. After 24 h, the percentages of T cells expressing CD154 were higher following stimulation with anti-CD3 and anti-CD28 antibodies and with CMV than in the negative control.

Supplementary Figure 2 | Distribution of CMV CD4+T cells from each of the three donors. **(A)** UMAP embeddings of CMV CD4+ T cells from each donor. Cells were assigned to each donor using demuxlet (28); ambiguous droplets were regarded as "unclear". Proportions of cells from each donor are shown on the left. UMAP embeddings were **(A)** colored or **(B)** split by donors. **(C)** Percentage of the five CMV-stimulated CD4+ cell clusters relative to total CD4+ T cells from each donor.

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