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Regulatory T cells in immune-mediated diseases

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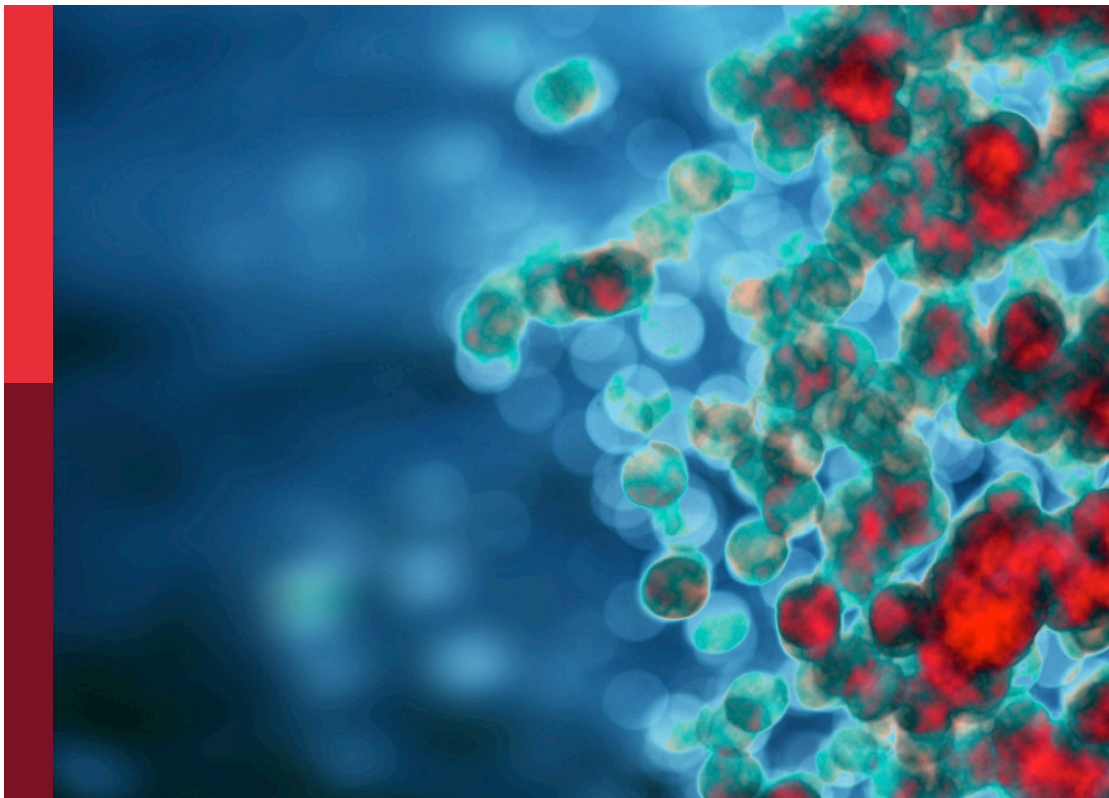
Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-8325-5892-8
DOI 10.3389/978-2-8325-5892-8

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Regulatory T cells in immune-mediated diseases

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Citation

Tran, G., Verma, N. D., Hall, B. M., Nicolls, M., eds. (2025). *Regulatory T cells in immune-mediated diseases*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-5892-8

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 23 December 2024
DOI 10.3389/fimmu.2024.1537314

OPEN ACCESS

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RECEIVED 30 November 2024
ACCEPTED 09 December 2024
PUBLISHED 23 December 2024

CITATION
Tran GT, Verma ND, Nicolls MR and Hall BM (2024) Editorial: Regulatory T cells in immune-mediated diseases.
Front. Immunol. 15:1537314.
doi: 10.3389/fimmu.2024.1537314

Editorial: Regulatory T cells in immune-mediated diseases

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KEYWORDS

Treg - regulatory T cell, immune mediated, immune suppression, Foxp 3, pTreg cells

Editorial on the Research Topic
Regulatory T cells in immune-mediated diseases

To ensure homeostasis, all biological systems exhibit both activating effects and regulatory mechanisms that counterbalance these effects. The discovery of regulatory and feedback mechanisms within immune responses has developed gradually over time. Thymic-derived T cells were first identified as inhibitory cells in 1970 (1) after the discovery of T cells by Miller in 1961 (2). Thymectomy in rodents shortly after birth led to the development of autoimmune responses (3), highlighting the regulatory role of the thymus. By the mid-1970s, research demonstrated that immune-mediated transplant tolerance, induced by neonatal infusion of allogeneic cells, could be transferred to a new host using enriched recirculating T cells (4). This observation initiated efforts to distinguish suppressor T cells from effector cells, an ongoing area of immunological investigation some of which is discussed in this Research Topic of papers and reviews.

During the 1970s and 1980s, research in murine models suggested the existence of $CD8^+$ $I\text{-}J^+$ T cells as suppressor cells. However, the inability to locate the I-J gene within the major histocompatibility complex (MHC) cast doubt on the existence of suppressor cells, leading to a substantial decline in investigations of suppressor T cells (5).

In a model of alloantigen-induced transplant tolerance in adult animals, it was observed that tolerance to specific alloantigen could be transferred by spleen and lymph node cells, as well as by enriched T cell preparations (6–9). Further characterization revealed that the tolerance-transferring T cells were $CD4^+$ rather than $CD8^+$. Unlike naive effector T cells, these $CD4^+$ T cells did not rapidly recirculate from blood to lymph (10, 11). Further investigation demonstrated that these tolerance-inducing T cells lost suppressor function ex vivo within days, unless stimulated by a specific alloantigen and a cytokine-rich supernatant derived from Con A-activated T cells (12, 13). IL-2 was one cytokine that promoted the survival of alloantigen specific tolerance inducing T regulatory cells. This led to the observation that $CD4^+CD25^+$ T cells, which express the interleukin-2 (IL-2) receptor, were essential for the transfer of alloantigen-specific tolerance (14). At that time, expression

of CD25 by activated T cells was considered a key marker of the rejection response and other. IL-2 was shown to partially sustain these tolerance mediating $CD4^+$ cells, suggesting other cytokines were involved in the maintenance of functional T regulatory cells.

Early studies identified additional markers, including Class II MHC and CD45RC (14), on alloantigen-specific T cells. These markers continue to aid in the identification of activated $CD4^+CD25^+$ T cells in both humans and animal models.

Sakaguchi and colleagues later demonstrated that $CD4^+CD25^+$ T cells in mice could prevent autoimmunity induced by thymectomy performed 3-4 days after birth (15). These cells, now termed thymus-derived regulatory T cells (tTregs), are involved in regulating immune responses broadly. When activated by an antigen, they selectively activate antigen-specific regulatory T cells (Tregs) (16).

More than fifty years after thymocytes were first described as suppressors of immune responses, and over thirty-five years since $CD4^+CD25^+$ T cells were identified as suppressor cells, the complexity and heterogeneity of $CD4^+CD25^+CD127^{lo}Foxp3^+$ T cells are still not fully elucidated. Continued research in this field will deepen our understanding of Tregs, particularly their activation and function, providing insights into immune regulation and potential therapeutic applications.

The articles in this Research Topic covers a wide-ranging research and reviews of the phenotypes and unique characteristics of Treg as well as its roles in various human diseases.

A new hypothesis for beneficial effects of polyvalent immunoglobulin G (IgG) in immune tolerance is proposed by Victor and Nahm through integration of the pre-existing idiotypic network theory and Treg cell theory into an anti-idiotypic Treg cell theory. The theory is based on demonstrated *in vitro* and *in vivo* effects of polyvalent IgG. These include increased IL-10 expression *in vitro* on $CD4^+CD25^+$Treg, increased IL-10 expressing $CD4^+$ cells in healthy donors, and increased $CD4^+Foxp3^+$cells in patients with atopic dermatitis associated with significant clinical improvement in patients. The proposed mechanism of IgG action involves multiple steps leading to IL-10 secretion by activated anti-idiotypic Treg through the presentation of immunogenic peptides generated from processing of IgG by dendritic cells. IL-10 in turn suppress Th2 cell response to allergens and autoimmune T cells response to self-antigens leading to IgG associated long-term clinical improvement in patients with allergic and autoimmune diseases. This theory needs validation by studying the detailed molecular mechanism underlying polyvalent IgG-induced Treg activation and examining its clinical usefulness.

Nirmala et al. conducted a detailed examination of various phenotypic markers to distinguish tTregs from pTregs and to identify activated tTregs. Liu et al., in contrast, characterized $FoxP3^+$ Tregs and found that both resting (or naive) $FoxP3^+$ Tregs and activated Tregs shared similar features, shedding light on the consistency of FoxP3 expression across Treg activation states.

Yilmazer et al. showed an unexpected high functional adaptability of peripheral Treg (pTreg) in absence of tTreg in mice model by selectively deleting either thymus-derived regulatory T cells (tTregs) or peripherally-induced regulatory T cells (pTregs). These pTregs acquire a highly activated suppressor phenotype and replenish the Treg cell pool. These observations contrast with the more established concept that tTregs are the primary mediators of self-tolerance. Overall, their study emphasized the important role of pTregs as mediators of self-tolerance and prompts further investigation of these cells for their potential use as a therapy for autoimmune diseases.

In a review on tissue resident regulatory T cells (TR-Tregs), Alvarez et al. provided insights about tissue residency program leading to generation of TR-Tregs. Inflammatory signals prior and during the migration of Tregs can alter the trajectory of these cells into tissues, leading them to adopt a helper T cell like phenotypes. Moreover, signals provided by other cytokines such as IL-12, IL-4 or IL-23 help them express T-bet^+, $GATA3^+$ or RORγ to express the appropriate Th chemokine receptor. This diverts T^+ effector Treg to migrate to inflammation sites alongside conventional T cells. Mechanisms that help maintain the specific phenotypes have been discussed. We have previously shown that IL-2 and alloantigen activated tTregs express IL12Rβ2 and are stimulated by IL-12 to mediate potent donor specific suppression *in vivo* (17). Like pTregs, a better mechanistic understanding TR-Treg *in vivo* biology can ultimately guide their future development as a cell-based therapy.

In experimental lupus-prone mice, Rosenberger et al. demonstrated that Tregs play a critical role in inhibiting autoreactive effector T cells. Their study showed a strong inverse correlation between the levels of Tregs and antigen-specific autoreactive $CD4^+$ T cells, emphasizing the direct role of Tregs in managing immune-mediated diseases.

In a comprehensive review, Xia et al. discussed the association between Tregs and cardiovascular diseases, presenting evidence from various studies that link Treg activity to cardiovascular health. They highlighted potential immunotherapies that may mitigate cardiovascular diseases by modulating Treg function.

Yilmaz et al. showed the role of Treg in pathogenesis of immune mediated liver disease, autoimmune liver disease (AILD) in scurfy mice and described features of disease pathogenesis. They confirmed that Treg deficiency is the key to spontaneous development of clinical, serological and immunopathological features of AILD. Wen et al. reviewed the role of T cells, mainly Th1/Th2 and Th17/Treg in pathogenesis of osteoarthritic (OA) and suggested future research directions for potentially new therapeutic strategies to include targeting and optimizing Th1/Th2 and Th17/Treg balance.

Zhang et al. investigated Treg populations in the blood of patients with sarcoidosis and found a significant correlation between sarcoidosis and a lower Treg population, alongside an elevated Th17.1 population. This imbalance suggests a dysregulation in immune cell populations in sarcoidosis patients.

Fukasawa et al. examined the efficacy of tildrakizumab, an IL-23 antibody, in patients with psoriasis. They found that clinical improvements were associated with enhanced Treg numbers and functionality, suggesting that tildrakizumab may exert therapeutic effects by promoting Treg-mediated immune regulation. Also, beneficial effects of supplements like glucosamine and chondroitin sulfate in repair and generation of chondrocytes need to be explored in detail. Vollmer et al. explored how leptin levels affect Treg proportions and functions in overweight individuals with

allergies, finding that leptin influences Treg activity in this population.

Angelats and Santamaria provided a comprehensive analysis of the transcription factors that govern the development of type 1 regulatory T (Tr1) cells and the regulatory mechanisms that define their lineage, offering insights into the distinct biological pathways of Tr1 cells.

Overall these studies enhance our understanding of Treg biology, including their phenotypes, activation mechanisms, and functional pathways. Such studies provide significant promise for treating autoimmune and inflammatory diseases. Continued research will develop targeted immunotherapies that harness the unique suppressive functions of Tregs.

Author contributions

GT: Writing – original draft, Writing – review & editing. NV: Writing – original draft, Writing – review & editing. MN: Writing – review & editing. BH: Writing – original draft, Writing – review & editing.

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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 13 July 2023
DOI 10.3389/fimmu.2023.1185443

OPEN ACCESS

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RECEIVED 13 March 2023
ACCEPTED 14 June 2023
PUBLISHED 13 July 2023

CITATION
Zhang H, Jiang D, Zhu L, Zhou G, Xie B, Cui Y, Costabel U and Dai H (2023) Imbalanced distribution of regulatory T cells and Th17.1 cells in the peripheral blood and BALF of sarcoidosis patients: relationship to disease activity and the fibrotic radiographic phenotype. *Front. Immunol.* 14:1185443. doi: 10.3389/fimmu.2023.1185443

Imbalanced distribution of regulatory T cells and Th17.1 cells in the peripheral blood and BALF of sarcoidosis patients: relationship to disease activity and the fibrotic radiographic phenotype

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Rationale: Sarcoidosis is a granulomatous interstitial lung disease involving a complex interplay among different cluster of differentiation 4 ($CD4^+$) thymus cell (T-cell) subsets. Originally described as a type 1 T-helper (Th1) inflammatory disease, recent evidence suggests that both effector and regulatory T-cell subgroups play a critical role in sarcoidosis, but this remains controversial.

Objectives: We aimed to investigate the distribution of $CD4^+$ T-cell subpopulations in sarcoidosis patients and its potential associations with clinical disease activity and a radiographic fibrotic phenotype.

Methods: We measured the frequencies of regulatory T cells (Tregs), Th1, Th17, and Th17.1 cells in the peripheral blood and/or bronchoalveolar lavage fluid (BALF) of 62 sarcoidosis patients, 66 idiopathic pulmonary fibrosis (IPF) patients, and 41 healthy volunteers using flow cytometry. We also measured the changes in these T-cell subpopulations in the blood at the follow-up visits of 11 sarcoidosis patients.

Measurements and results: An increased percentage of Tregs was observed in the peripheral blood of sarcoidosis patients, with a positive association to disease activity and a fibrotic radiographic phenotype. We found a higher frequency of Tregs, a lower proportion of Th17.1 cells, and a lower ratio of Th17.1 cells to total Tregs in the peripheral blood of both active and fibrotic sarcoidosis patients, compared with IPF patients or healthy donors. In contrast, a lower frequency of Tregs and a higher proportion of Th17.1 cells was found in the BALF of sarcoidosis

patients than in that of IPF patients. There was an imbalance of Tregs and Th17.1 cells between the peripheral blood and BALF in sarcoidosis patients. Following immunoregulatory therapy, the proportion of circulating Tregs in sarcoidosis patients decreased.

Conclusion: A higher proportion of Tregs in the peripheral blood of sarcoidosis patients was related to disease activity, fibrotic phenotype, and the need for immunoregulatory therapy. The imbalanced distribution of Tregs and Th17.1 cells in patients' peripheral blood and BALF suggests that the lung microenvironment has an effect on the immunological pathogenesis of sarcoidosis. Therefore, further studies on the functional analysis of Tregs and Th17.1 cells in sarcoidosis patients are warranted.

KEYWORDS

sarcoidosis, regulatory T cells, Th17.1 cells, peripheral blood, bronchoalveolar lavage fluid

Introduction

Sarcoidosis is a multisystemic inflammatory disorder of unknown etiology, which is characterized by the presence of non-caseating granulomas, and predominantly affects the lungs (1). Patients with sarcoidosis show a wide range of clinical presentations, natural disease course, and disease outcome; this can be attributed to individuals' genetic predisposition and environmental factors (2). The majority of sarcoidosis patients reach remission with or even without treatment, whereas a minority develop chronic and sometimes progressive disease, even leading to death (3). However, until now, no single biomarker has been identified as being useful in establishing a definite diagnosis of sarcoidosis and/or predicting the disease outcome (4, 5). The exact pathogenesis of sarcoidosis remains incompletely understood, but a cluster of differentiation 4 ($CD4^+$) T-cell-induced immune response is generally acknowledged to be a key player in the maintenance of the granulomatous inflammation (6, 7).

Recent advances in better understanding the function of diverse immune cells in sarcoidosis suggest, besides $CD4^+$ T cells, a critical role of effector and regulatory T-cell subgroups in the pathogenesis of this disorder (8). This complements the original concept of sarcoidosis as a disease driven by a Th1-dominant immune response, with abundant cytokine expression of interferon-gamma (IFN-γ), interleukin (IL)-2, IL-12, and IL-18 (9–12). Emerging evidence indicates that Th17 cells associated with enhanced IL-17A expression play an important role in sarcoidosis, as they do in many other autoimmune diseases (13–15). A subset of Th17 cells, called Th17.1 cells, is characterized by its capacity to produce both IL-17 and IFN-γ, apparently combining the functional properties of Th17 cells and Th1 cells (16–18). It is still unclear, however, whether Th17.1 cells play a protective or pathogenic role in sarcoidosis. In regard to their association with a favorable prognosis or a chronic disease course, two studies have reported discordant results (19, 20). Reports on the role of regulatory T cells (Tregs) in sarcoidosis are also conflicting, with the cells being reported as having both increased and decreased frequencies (21–23). Furthermore, an elevated percentage of Tregs, but with an impaired immunosuppressive capacity, has also been observed in sarcoidosis patients (24, 25). Intriguingly, it has also been postulated that an imbalance of Th17 cells and Tregs is responsible for disease progression (26).

In this study, we aimed to investigate the distribution of $CD4^+$ effector and regulatory T-cell subpopulations in sarcoidosis patients in more detail and analyze their association with disease activity and radiographic phenotype. For this purpose, we used flow cytometry to measure the levels of Tregs, Th1, Th17, and Th17.1 cells in the peripheral blood and/or the bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis or idiopathic pulmonary fibrosis (IPF), and of healthy volunteers.

Materials and methods

Study design and participants

We consecutively enrolled 62 participants with sarcoidosis who had been diagnosed at the Department of Pulmonary and Critical Care Medicine, China-Japan Friendship Hospital, from January 2019 to January 2021. The diagnosis of sarcoidosis was confirmed through multidisciplinary discussion (MDD), based on compatible clinical and chest CT findings, together with the presence of non-necrotizing granulomatous inflammation, and the exclusion of alternative causes of granulomatous disease, consistent with the statement from the American Thoracic Society (ATS)/European Respiratory Society (ERS)/World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) (1). Exclusion criteria

were acute respiratory tract infection, concomitant pulmonary disease [including chronic obstructive pulmonary disease (COPD) and asthma], autoimmune diseases, malignancies, organ transplantation, and allergies. The disease activity was defined as follows: (a) recently developed or increasing symptoms such as cough, dyspnea, weakness, fever, and arthralgia; and/or (b) evidence of progressive disease on chest CT; and/or (c) test results indicating the deterioration of lung function; and/or (d) abnormal laboratory test results, such as high levels of serum angiotensin-converting enzyme (sACE), and lymphocytosis with an increased ratio of $CD4^+/CD8^+$ cells in BALF (1, 27, 28). The study design did not require individuals to be newly diagnosed or treatment naive. The treatments given included glucocorticoids, methotrexate, azathioprine, mycophenolate, and biological agents. Follow-up visits were conducted 3 months after enrollment in the study.

The disease control group consisted of 66 individuals who had been diagnosed with IPF. All participants met the international diagnostic criteria for IPF; for this group, we used the same exclusion criteria that had been used for the sarcoidosis patient group (29).

The 41 healthy individuals underwent health check-ups, wherein no chest radiographic abnormalities and no history of malignancies, autoimmune diseases, allergies, or other lung disorders were identified.

The Research Review Committee and the Ethical Review Committee of the China-Japan Friendship Hospital approved this study. Every participant provided written informed consent to participate in the study.

Peripheral blood mononuclear cells and bronchoalveolar lavage

The fasting peripheral blood was taken within 2 days of bronchoalveolar lavage (BAL) being carried out for sarcoidosis and IPF patients. For those patients who did not undergo bronchoscopy at the baseline or at follow-up visits, and for healthy volunteers, fasting blood was drawn. Whole blood was then collected in sodium heparin tubes. Peripheral blood mononuclear cells (PBMCs) were then isolated using Ficoll-Paque™ Plus (GE Healthcare) density gradient separation followed by washing twice with phosphate-buffered saline (PBS; Hyclone), as previously described (30).

BAL was conducted during bronchoscopy after the administration of local anesthesia and before tissue biopsies, as previously described (31). The choice of the lavage site was guided by the location of the parenchymal pathology on high-resolution computed tomography (HRCT), and, if it was diffuse, the right middle lobe was chosen. With the bronchoscope in a wedged position in a segmental bronchus, 100 mL of sterile saline solution (0.9% NaCl) was decanted into aliquots of 30 mL, 30 mL, 40 mL, and another 40 mL was decanted if necessary. BALF was retrieved by applying a gentle suction into a silicone-treated bottle, and was stored at 4°C until processing within 4 hours of collection. BALF was filtered through a 40-μm cell strainer (BD Biosciences) and centrifuged. Erythrocytes were eliminated using lysing buffer (BD Biosciences) and washing with PBS.

Both PBMCs and BAL cells were frozen in 1 mL of fetal bovine serum (FBS; Gibco) with 10% dimethyl sulfoxide (DMSO; Sigma) in a cryovial using a 5100 Cryo 1°C Freezing Container (Nalgene) to 80°C, and, thereafter, stored in a liquid nitrogen biorepository.

Flow cytometry

PBMCs and BAL cells were analyzed using an LSRFortessa™ flow cytometer (BD Biosciences) by employing an eight-parameter flow cytometry panel. Frozen cells were thawed, resuspended in PBS, and stained on ice in the dark. Staining with Fixable Viability Stain 510 (BD Horizon™) at a 1 : 1,000 dilution in PBS was carried out for the assessment of cell viability and staining with Fc Block™ (BD Pharmingen™) was conducted to prevent non-specific staining. For surface staining, we incubated cells in Brilliant Stain Buffer (BD Horizon) with antibodies per the manufacturer's instructions, including CD3 PerCP-eFluor 710 (Invitrogen), CD4 BUV395 (BD Horizon), CD45RA BV605 (BD Horizon), CXCR3 (CD183) BV786 (BD OptiBuild™), CCR4 (CD194) PE-CF594 (BD Horizon), and CCR6 (CD196) BB515 (BD Horizon). For intracellular staining, we used the forkhead box protein P3 (FOXP3) Alexa Fluor® 647 antibody (BD Pharmingen), in addition to the Transcription Factor Buffer Set (BD Pharmingen) for both fixation and permeabilization. The flow cytometry characteristic definitions of Tregs, Th1, Th17, and Th17.1 cells are listed in Table 1. FlowJo v10.7 software (BD) was used to analyze the flow cytometric data and the gating strategy is shown in Figure 1.

Statistical analysis

SPSS Statistics version 16.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA)

TABLE 1 $CD4^+$ T-cell subset phenotypes.

$CD4^+$ T-cell subset	FOXP3	CD45RA	*CXCR3*	*CCR4*	*CCR6*
Tregs	+	/	/	/	/
Th1	−	−	+	−	−
Th17	−	−	−	+	+
Th17.1	−	−	+	−	+

Th1, T helper type 1; Tregs, regulatory T cells. +, positively stained; −, negatively stained;/, no matter how the subsequent staining.

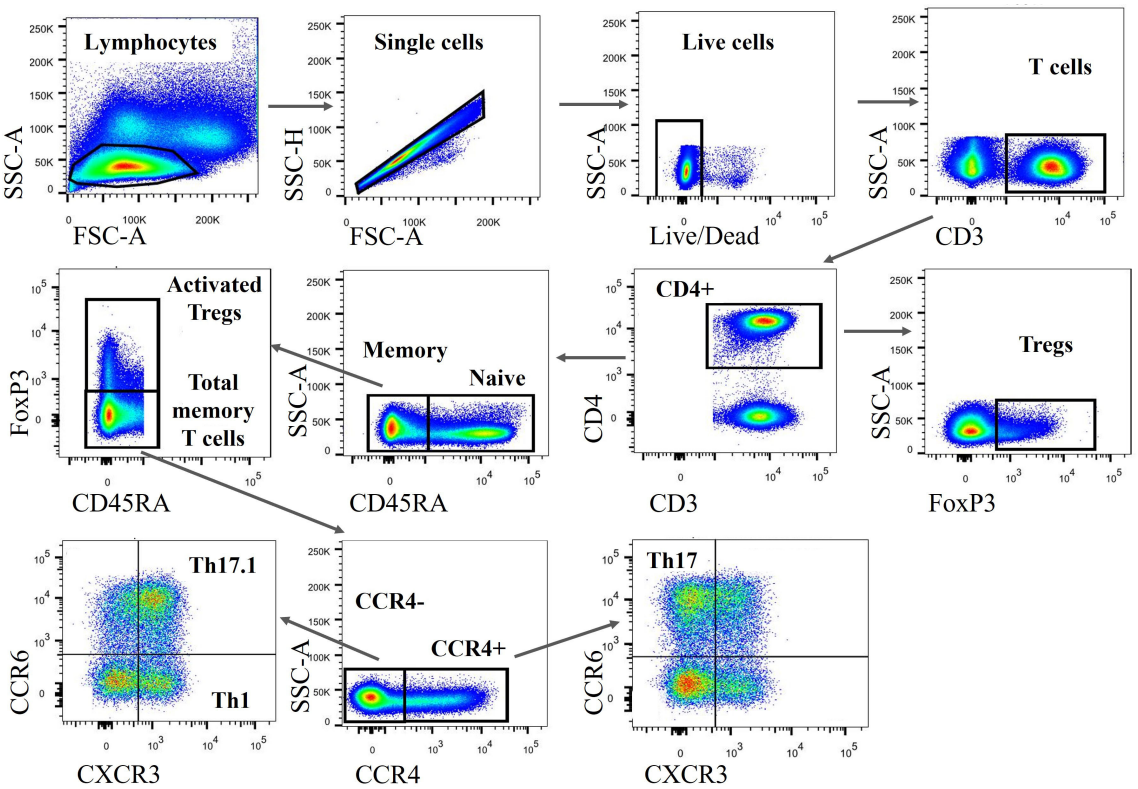

FIGURE 1
Gating strategy to identify various $CD4^+$ T-cell subsets. Both PBMCs and BAL cells were subsequently gated on lymphocytes (based on FSC-A vs. SSC-A), single cells (based on FSC-A vs. SSC-H), live cells (defined as Fixable Viability Stain 510 negatively stained cells), $CD3^+$, and $CD4^+$ cells. Subsequently, $FOXP3^+$ Tregs, $FOXP3^+$ CD45RA- activated Tregs and $FOXP3^-$ $CD45RA^-$ total memory T cells were identified. Stepwise, we classified those total memory T cells into different subgroups, including Th1, Th17, and Th17.1 cells based on the expression of *CCR4*, *CXCR3*, and *CCR6*. PBMCs, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; $CD4^+$, cluster of differentiation 4; Th1, T helper type 1; FSC-A, forward scatter area; SSC-A, side scatter area; SSC-H, side scatter height; CXCR3, chemokine (C-X-C motif) receptor 3; CXR4, chemokine (C-C motif) receptor 4..

were used. Comparisons between the groups were assessed by way of an unpaired Student's *t*-test, the Mann–Whitney *U*-test, chi-squared analysis, or a one-way ANOVA test, as appropriate. A paired Student's *t*-test was used for the paired comparison. Pearson or Spearman rank correlation tests were used to investigate correlations. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Characteristics of participants

There were 62 patients with sarcoidosis, 66 patients with IPF, and 41 healthy volunteers included in this study (Table 2). The mean age of sarcoidosis patients was 52 years; this was lower than that of IPF patients, but not different from that of the healthy donors. Sarcoidosis patients were predominantly female and non-smokers. Sarcoidosis patients had a higher proportion of lymphocytes and a higher ratio of $CD4^+/CD8^+$ cells in their BALF compared with IPF patients. There were 25 patients in the active sarcoidosis group, and 37 in the inactive group. Nearly one-third (20/62) of sarcoidosis patients had extrapulmonary organ involvement, including peripheral lymph nodes, skin, eyes and others.

The proportion of circulating Tregs in sarcoidosis patients is associated with disease activity and fibrotic radiographic stages

The flow cytometric analysis of the proportion of diverse $CD4^+$ T-cell subsets in the blood of sarcoidosis patients showed that Tregs, Th1, Th17, and Th17.1 cells constituted 7.1% ± 0.7%, 3.0% ± 0.3%, 10.25% ± 0.6%, and 3.15% ± 0.3%, of CD4+ T cells, respectively (Figure 2A). The proportion of Tregs in patients with active sarcoidosis was higher than in those with inactive sarcoidosis (7.95% ± 1.5% vs. 5.55% ± 0.4% of $CD4^+$ T cells; $p = 0.001$) (Figures 2B–D). The proportion of Tregs correlated positively with levels of sACE and the ratio of $CD4^+/CD8^+$ cells in patients' BALF ($r = 0.463$, $p < 0.001$; and $r = 0.486$, $p = 0.003$) (Figures 2E, F). Patients with fibrotic radiographic stages had a higher proportion of Tregs than those with non-fibrotic stages (8.4% ± 3.1% vs. 6.05% ± 0.4% of $CD4^+$ T cells; $p = 0.045$) (Figures 2G–I).

TABLE 2 Demographics and clinical characteristics.

Characteristic	Sar (n = 62)	IPF (n = 66)	HC (n = 41)
Age (years)	52 ± 13	66 ± 8 $^{\Pi}$	48 ± 9
Male/female	15/47	59/7	7/34
Current smoker/former smoker/never smoked	6/5/51	8/40/18	4/2/35
Sarcoidosis stage I/II/III/IV	7/43/7/5		
Sarcoidosis active/inactive	25/37		
Sarcoidosis fibrotic/non-fibrotic	12/50		
Airway mucous nodule	20		
Extrapulmonary organ involvement	20		
Immunoregulatory medication	17		
sACE (U/L)	47.3 ± 31.2		
CD4/CD8 ratio in BALF	6.6 ± 8.2	1.0 ± 0.7 $^{\Pi}$	
Lymphocytes in BALF(%)	34.8 ± 39.8	9.0 ± 10.2 $^{\Pi}$	
FEV_1%/pre	92.4 ± 20.8	82.2 ± 18.3 $^{\Pi}$	
FVC%/pre	103.3 ± 20.6	78.8 ± 18.5 $^{\Pi}$	
TLC%/pre	90.3 ± 15.6	68.2 ± 14.7 $^{\Pi}$	
$DLCO_{SB}$%/pre	81.9 ± 19.3	50.1 ± 18.2 $^{\Pi}$	
6MWD/m	499.9 ± 69.7	468.3 ± 89.6	

Data are shown as mean ± SEM or number.
Chest radiography staging was based on the Scadding stage criteria.
$^{\Pi}$: statistic difference ($p < 0.05$) between sarcoidosis and IPF patients.
Sar, sarcoidosis; IPF, idiopathic pulmonary fibrosis; HC, healthy control; sACE, serum angiotensin-converting enzyme; BALF, bronchoalveolar lavage fluid; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; TLC, total lung capacity; DLCO, carbon monoxide diffusing capacity; 6MWD, 6-minute-walk distance.

Higher percentage of circulating Tregs and lower proportion of Th17.1 cells in active and fibrotic sarcoidosis patients

We found an increased proportion of circulating Tregs in active sarcoidosis patients compared with patients with inactive sarcoidosis or IPF or healthy volunteers (7.95% ± 1.5% vs. 5.5% ± 0.4% vs. 6.7% ± 0.3% vs. 5.1% ± 0.4%; $p < 0.001$, $p = 0.004$, and $p < 0.001$, respectively) (Figure 3A). We found that both active and inactive sarcoidosis patients had lower proportions of Th17.1 cells than IPF patients (3.4% ± 0.5% and 2.9% ± 0.4% vs. 5.6% ± 0.5%; $p = 0.002$ and $p < 0.001$, respectively) (Figure 3B). The percentage of Th17.1 cells was also lower in inactive sarcoidosis patients than in healthy donors (4.4% ± 0.3%; $p = 0.031$) (Figure 3B). Moreover, the ratio of Th17.1 cells to total Tregs was lower for both active and inactive sarcoidosis patients than for IPF patients or healthy volunteers (0.5 ± 0.1 and 0.6 ± 0.1 vs. 0.9 ± 0.1 and 1.1 ± 0.1; $p = 0.003$ and $p < 0.001$; $p = 0.021$ and $p = 0.002$, respectively) (Figure 3C). Likewise, the proportion of Tregs was higher in fibrotic sarcoidosis patients than in non-fibrotic sarcoidosis, IPF patients, or healthy participants (8.4% ± 3.1% vs. 6.0% ± 0.4%, vs. 6.4% ± 0.3%, vs. 5.1% ± 0.4%; $p = 0.005$, $p = 0.009$, and $p < 0.001$, respectively) (Figure 3D). However, the percentage of Th17.1 cells for both fibrotic and non-fibrotic sarcoidosis patients was lower than that of IPF patients (2.1% ± 0.4% vs. 3.3% ± 0.4% and vs. 5.6% ± 0.5%; $p < 0.001$ and $p < 0.001$, respectively) (Figure 3E). We found that there was a lower proportion of Th17.1 cells in fibrotic sarcoidosis patients than in healthy controls (4.4% ± 0.3%; $p = 0.023$) (Figure 3E). Consequently, the ratio of Th17.1 cells to total Tregs was decreased for not only fibrotic but also non-fibrotic sarcoidosis patients, compared with IPF patients or healthy individuals (0.4 ± 0.1 and 0.6 ± 0.1 vs. 0.9 ± 0.1 and 1.1 ± 0.6; $p = 0.006$ and $p = 0.001$; $p = 0.009$ and $p = 0.001$, respectively) (Figure 3F).

Decreased percentage of Tregs and increased proportion of Th17.1 cells in BALF from sarcoidosis patients compared with IPF patients

Tregs, Th1, Th17, and Th17.1 cells constituted 5.7% ± 0.7%, 4.0% ± 0.6%, 12.7% ± 2.6%, and 27.7% ± 3.1% of $CD4^+$ T cells in the BALF of sarcoidosis patients, respectively (Figure 4A). The percentage of Tregs in the BALF of both active and inactive sarcoidosis patients was significantly lower than in IPF patients (5.2% ± 0.7% and 6.9% ± 1.7% vs. 23.0% ± 3.4%; $p < 0.001$ and $p < 0.001$, respectively) (Figure 4B). In addition, the percentage of Th17.1 cells was significantly higher than in IPF patients (28.4% ± 4.0% and 26.3% ± 5.1% vs. 3.4% ± 1.1%; $p < 0.001$ and $p = 0.001$, respectively)

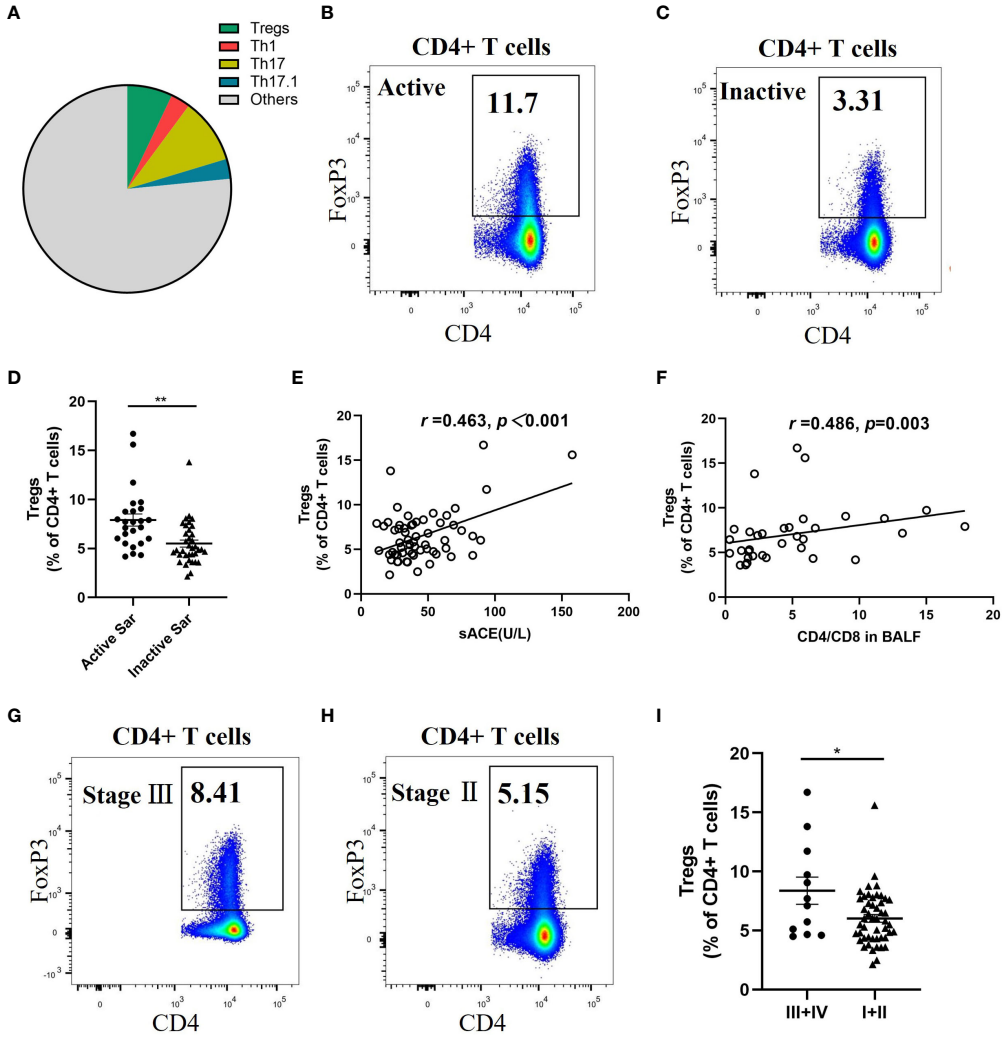

FIGURE 2
Significant increase of circulating Tregs proportion in active and fibrotic sarcoidosis patients. **(A)** Pie chart showing the mean percentage of Tregs, Th1, Th17, Th17.1, and other unmentioned $CD4^+$ T cells in the peripheral blood of sarcoidosis patients. **(B, C)**. Flow cytometry dot plot of Tregs gated by the expression of FOXP3 from an active and an inactive sarcoidosis patient, respectively. **(D)** Comparison of the proportion of circulating Tregs between active and inactive sarcoidosis patients (7.9% ± 1.5% vs. 5.5% ± 0.4%; $p = 0.001$). **(E, F)** Positive correlations between the proportion of Tregs and the sACE level ($n = 60$) and the ratio of $CD4^+/CD8^+$ cells in BALF ($n = 34$) of sarcoidosis patients ($r = 0.463$, $p < 0.001$; $r = 0.486$, $p = 0.003$). **(G, H)** Flow cytometry dot plot of Tregs from a stage III and a stage II sarcoidosis patient, respectively. **(I)** Comparison of the proportion of circulating Tregs between fibrotic (stage III/IV) and non-fibrotic (stage I/II) sarcoidosis patients (8.4% ± 3.1% vs. 6.0% ± 0.4%; $p = 0.045$). Data are expressed as mean ± SEM. An unpaired Student's *t*-test was used to compare the proportion of circulating Tregs in active sarcoidosis patients with that in inactive sarcoidosis patients, and that in fibrotic patients with that in non-fibrotic sarcoidosis patients. Pearson and Spearman rank correlation tests were used to investigate correlation. $*p < 0.05$; $**p < 0.01$. BALF, bronchoalveolar lavage fluid; $CD4^+$, cluster of differentiation 4; sACE, serum angiotensin converting enzyme; Th1, T helper type 1; Tregs, regulatory T cells.

(Figure 4C). Consequently, the ratio of Th17.1 cells to total Tregs in BALF was higher in patients with active sarcoidosis than in those with IPF (8.5 ± 2.4 vs. 0.2 ± 0.1; $p = 0.004$) (Figure 4D).

Imbalanced distribution of Tregs and Th17.1 cells in the peripheral blood and BALF of sarcoidosis patients

It was interesting to note that the distribution frequency of Th17.1 cells was remarkably high in the BALF of sarcoidosis patients, whereas it was barely detectable in their peripheral blood (Figures 5A, B). The proportion of Th17.1 cells was much higher in the BALF than in the peripheral blood of sarcoidosis patients ($p < 0.0001$) (Figure 5C). However, there was no difference in the percentage of Tregs found in the BALF and blood of sarcoidosis patients (Figure 5D). Consequently, the ratio of Th17.1 cells to total Tregs in BALF was markedly increased, compared with the peripheral blood of sarcoidosis patients ($p = 0.001$) (Figure 5E). Both the increased proportion of Th17.1 cells in lungs and the higher ratio of Th17.1 cells to total Tregs in the BALF were observed only in sarcoidosis patients and not in IPF patients (Figures 5C, E).

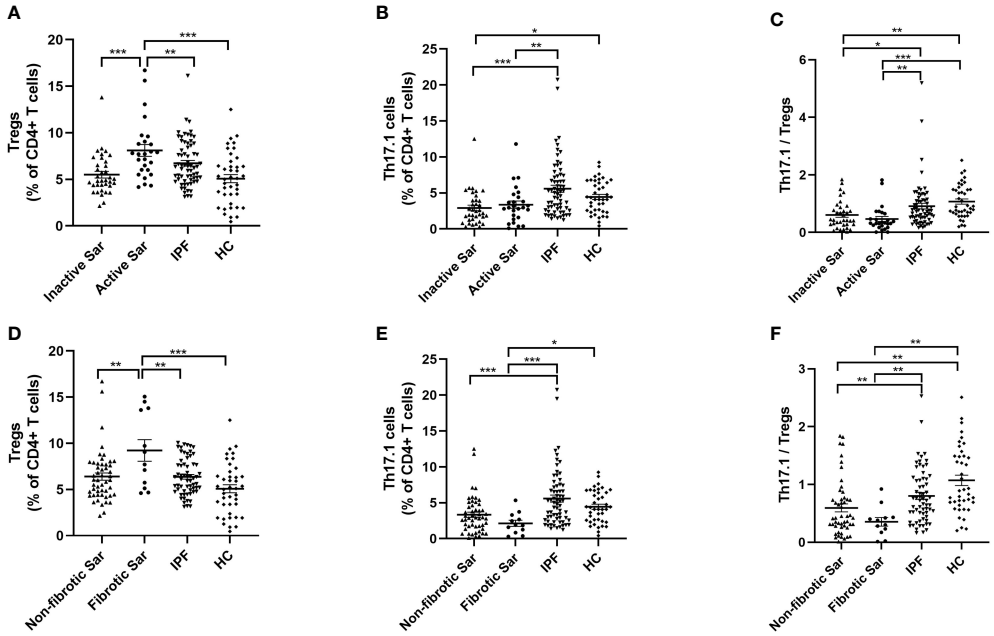

FIGURE 3
Comparative analysis of the distribution of Tregs and Th17.1 cells in the peripheral blood of sarcoidosis and IPF patients and healthy volunteers. **(A)** The proportion of circulating Tregs of active sarcoidosis patients ranked first, rather than that of inactive sarcoidosis or IPF or HC subjects (7.9% ± 1.5% vs. 5.5% ± 0.4%, vs. 6.7% ± 0.3%, vs. 5.1% ± 0.4%; $p < 0.001$, $p = 0.004$, and $p < 0.001$). **(B)** Both active and inactive sarcoidosis patients had a lower proportion of Th17.1 cells than IPF patients (3.4% ± 0.5% and 2.9% ± 0.4% vs. 5.6% ± 0.5%; $p = 0.002$ and $p < 0.001$). The percentage of Th17.1 cells was also lower in inactive sarcoidosis patients than in healthy volunteers (4.4% ± 0.3%; $p=0.031$). **(C)** The ratio of Th17.1 cells to total Tregs in both active and inactive sarcoidosis patients was lower than that in IPF patients or healthy donors (0.5 ± 0.1 and 0.6 ± 0.1 vs. 0.9 ± 0.1 and 1.1 ± 0.1; $p = 0.003$ and $p < 0.001$; $p = 0.021$ and $p = 0.002$). **(D)** The proportion of Tregs in fibrotic sarcoidosis patients was higher than that in non-fibrotic sarcoidosis, IPF, or HC subjects (8.4% ± 3.1% vs. 6.0% ± 0.4%, vs. 6.4% ± 0.3%, vs. 5.1% ± 0.4%; $p = 0.005$, $p = 0.009$, and $p < 0.001$). **(E)** Both fibrotic and non-fibrotic sarcoidosis patients had a lower proportion of Th17.1 cells than IPF patients (2.1% ± 0.4% vs. 3.3% ± 0.4% and 5.6% ± 0.5%; $p<0.001$ and $p<0.001$). The percentage of Th17.1 cells was also lower in fibrotic sarcoidosis patients than that in healthy donors (4.4% ± 0.3%; $p=0.023$). **(F)** The ratio of Th17.1 cells to total Tregs in both fibrotic and non-fibrotic sarcoidosis patients was lower than that in patients with IPF or HC (0.4 ± 0.1 and 0.6 ± 0.1 vs. 0.9 ± 0.1 and 1.1 ± 0.6; $p = 0.006$ and $p = 0.001$; $p = 0.009$ and $p = 0.001$). One-way ANOVA test was used for data analysis. *$p < 0.05$; **$p < 0.01$; ***$p <0.001$. Sar, sarcoidosis; IPF, idiopathic pulmonary fibrosis; HC, healthy control; Tregs, regulatory T cells; Th17.1, T helper type 17.1.

In contrast, a significantly higher proportion of Treg cells was observed in the BALF than the blood of IPF patients, but this was not the case for sarcoidosis patients ($p = 0.005$) (Figure 5D).

Decreased fraction of circulating Tregs by immunoregulatory therapy for sarcoidosis patients

A total of 17 sarcoidosis patients had been taking immunoregulatory medication 3 months prior to their enrolment in the study, and they had lower proportions of circulating Tregs than those who had not been taking immunoregulatory medication ($p = 0.019$) (Figure 6A). In total, 11 sarcoidosis patients, who took glucocorticoids, were followed up for 3 months after the study. The distribution frequency of the circulating Tregs of these patients at the 3-month follow-up visit decreased significantly, compared with the baseline visit ($p = 0.022$) (Figure 6B). As for the proportion of Th17.1 cells and the ratio of Th17.1 cells to total Tregs, there was no difference when comparing those patients measured at the 3-month follow-up visit with those measured at the baseline visit ($p = 0.513$; $p = 0.262$) (Figures 6C, D).

Discussion

The results of this study on the distribution of $CD4^+$ T-cell subpopulations revealed that there was an increased frequency of Tregs in the blood, and of Th17.1 cells in the BALF, of sarcoidosis patients. This increased frequency of circulating Tregs was associated with disease activity and a fibrotic radiographic phenotype.

Tregs exhibit strong immunosuppressive capacities on various immune cells. The role that Tregs play in sarcoidosis remains controversial. Early reports claimed that the frequency of FOXP3-expressing blood Tregs was decreased in sarcoidosis patients compared with control participants (21). The expression of FOXP3, however, was markedly increased after corticosteroid medication (25). The majority of the studies in this field have found that an increased frequency of Tregs is usually observed in the active phase of this disease (23, 24), and our results are in line with this. Miyara et al. (23) found that $CD4^+$ $CD25^{bright}$ $FOXP3^+$ Tregs accumulated in the peripheral blood and that there was a reduction in the frequency of circulating Tregs on resolution of sarcoidosis. The same research group showed, beside the amplification of blood Tregs in active sarcoidosis, that $CD45RA^-$ $FOXP3^{BRIGHT}$ Tregs proliferated and accumulated with granulomas

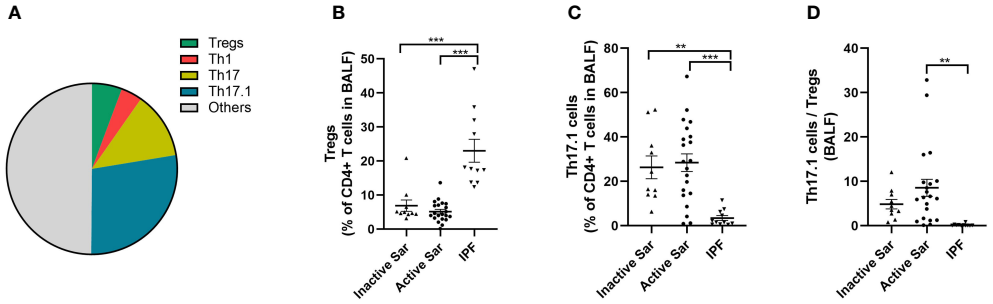

FIGURE 4
Comparative analysis of the distribution of Tregs and Th17.1 cells in BALF among active or inactive sarcoidosis and IPF patients. **(A)** Pie chart showing the mean percentage of Tregs, Th1, Th17, Th17.1, and other unmentioned $CD4^+$ T cells in the BALF of sarcoidosis patients. **(B)** We found a lower proportion of Tregs in the BALF of both active and inactive sarcoidosis patients than in that of IPF patients (5.2% ± 0.7% and 6.9% ± 1.7% vs. 23.0% ± 3.4%; $p < 0.001$ and $p < 0.001$). **(C)** The percentage of BALF Th17.1 cells in both active and inactive sarcoidosis patients was higher than that in IPF patients (28.4% ± 4.0% and 26.3% ± 5.1% vs. 3.4% ± 1.1%; $p < 0.001$ and $p = 0.001$). **(D)** The ratio of Th17.1 cells to total Tregs in the BALF of active sarcoidosis patients was significantly higher than that in IPF patients (8.5 ± 2.4 vs. 0.2 ± 0.1; $p = 0.004$). One-way ANOVA test was used for data analysis. $^{**}p < 0.01$; $^{***}p<0.001$. BALF, bronchoalveolar lavage fluid; Sar, sarcoidosis; IPF, idiopathic pulmonary fibrosis; Tregs, regulatory T cells; Th17.1, T helper type 17.1.

(24). They found no correlation between the percentage of Tregs and sACE level, but a positive association to the extent of renal interstitial fibrosis (24). In patients with pulmonary sarcoidosis, an increased proportion of Tregs at the time of diagnosis was observed in patients developing chronic disease during follow-up (32). In our study, we found that there was a correlation between the proportion of circulating Tregs, sACE, and the $CD4^+/CD8^+$ cell ratio in the BALF, and patients with a fibrotic radiographic stage had higher proportion of circulating Tregs. We also showed in our study that sarcoidosis patients who had been taking immunoregulatory medication 3 months prior to study enrolment had a lower frequency of circulating Tregs than those who had not been taking such medication. Moreover, we showed that the frequency of circulating Tregs in sarcoidosis patients who were treated with

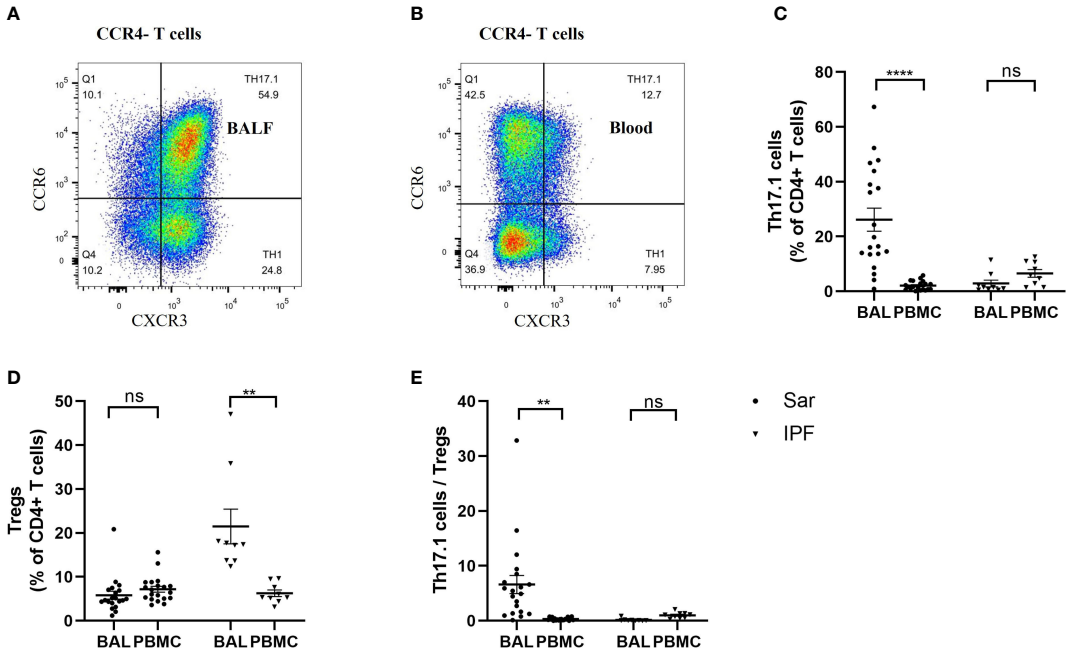

FIGURE 5
Comparison of the frequency of Th17.1 cells and proportion of Tregs between the BALF and peripheral blood in sarcoidosis ($n = 20$) and IPF ($n = 9$) patients. **(A, B)** Flow cytometry dot plot of Th17.1 cells gated by the expression of CXCR3 and CCR6 in $CCR4^-$ T cells from BALF and peripheral blood of a sarcoidosis patient, respectively. **(C)** There was a significant difference in the percentage of Th17.1 cells found in the BALF and peripheral blood in sarcoidosis patients (26.2% ± 4.2% vs. 2.1% ± 0.4%; $p < 0.0001$). **(D)** We found that sarcoidosis patients had a similar proportion of Tregs in their BALF and peripheral blood. **(E)** The ratio of Th17.1 cells to total Tregs of sarcoidosis patients was higher in BALF than in peripheral blood (6.6 ± 1.7 vs. 0.3 ± 0.1; $p=0.001$). A paired Student's t-test was conducted for the paired comparison. $^{**}p < 0.01$; $^{****}p < 0.0001$; ns, no significance. BALF, bronchoalveolar lavage fluid; PBMC, peripheral blood mononuclear cell; Sar, sarcoidosis; IPF, idiopathic pulmonary fibrosis; Tregs, regulatory T cells; Th17.1, T helper type 17.1.

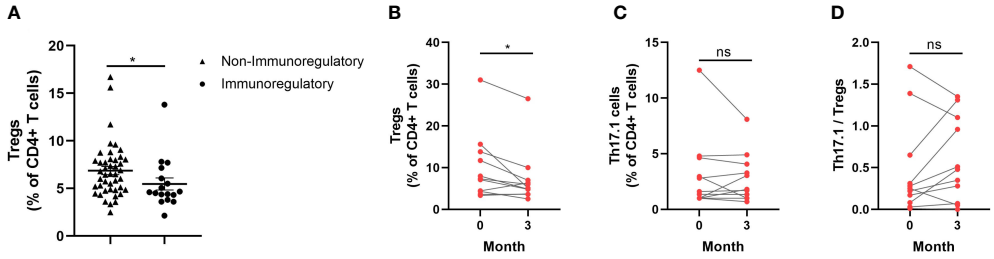

FIGURE 6
Changes of circulating Tregs proportion by immunoregulatory therapy for sarcoidosis patients. **(A)** Comparison of circulating Tregs proportion in patients who were taking with those not taking immunoregulatory medication (median 4.6% vs. 6.5%; $p = 0.019$). **(B)** The proportion of Tregs reduced so as to be different to a statistically significant degree 3 months after study enrolment in patients treated with glucocorticoids when compared with that measured at the baseline visit (median 5.1% vs. 7.3%; $p = 0.022$). **(C, D)** There was no difference in the proportion of Th17.1 cells and the ratio of Th17.1 cells to total Tregs at the 3-month follow-up visit, compared with the measurement at the baseline visit. An unpaired Student's *t*-test was used to compare the proportion of circulating Tregs in patients who were taking immunoregulatory medication with those who were not. A paired Student's t-test was conducted in the period between the baseline and follow-up visits. $*p < 0.05$; ns, no significance.

corticosteroids after diagnosis was significantly decreased at the 3-month follow-up.

Despite their being present in large numbers, Tregs from sarcoidosis patients have been found to be functionally incompetent (33). Sarcoidosis patient-derived Tregs demonstrated a weaker capacity for inhibiting inflammatory cytokine production than Tregs obtained from healthy donors, and Tregs regained their suppressive capacity with disease resolution (34). Broos et al. (32) found that circulating Tregs were apoptotic-prone owing to their overexpression of CD95, leading to their impaired survival. In another study, it was reported that the repressor activity was impaired only in BALF Tregs and not in the peripheral blood Tregs of sarcoidosis patients, taking telomere length and the ability to produce transforming growth factor beta (TGF-β) and IL-10 into account (25). A functional analysis of Tregs was not conducted in our study, and this is an important limitation of our research.

A minority of sarcoidosis patients will develop chronic, and some even progressive disease, complicated by pulmonary hypertension or pulmonary fibrosis (3). There are some similarities between fibrotic sarcoidosis and IPF, the prototype of a progressive fibrotic interstitial lung disease (ILD). In this study, patients with IPF were used as disease controls. We found that there was a significantly higher frequency of circulating Tregs in active or fibrotic sarcoidosis patients than in patients with IPF. Regrettably, few fibrotic sarcoidosis patients donated BALF cells, as the majority of patients were either intolerant to or unwilling to undergo bronchoscopy, meaning that we were unable to compare the activity of lung Tregs in fibrotic patients with that in non-fibrotic sarcoidosis patients.

It has recently been recognized that Th17.1 cells play a role in the pathogenesis of sarcoidosis. Tøndell et al. (22) reported that the proportion of IFN-γ^+ Th17.1 cells in BALF was higher in sarcoidosis patients than in healthy participants, and there was no significant difference between the proportion of these cells in sarcoidosis patients and in patients with other ILDs, including hypersensitivity pneumonitis (HP), IPF, connective tissue disease-associated ILD (CTD-ILD), and an unspecified ILD, and an increased ratio of lung Th17.1 cells to Tregs in sarcoidosis patients, compared with patients with other ILDs or healthy participants. Similarly, our study demonstrated that a significantly higher proportion of Th17.1 cells and an increased ratio of Th17.1 cells to Tregs in BALF were present in sarcoidosis patients compared with IPF patients. Ramstein et al. reported, in line with our data, that Th17.1 cells constituted approximately 30% of the lung $CD4^+$ T cells and that their frequency was markedly higher in the BALF of sarcoidosis patients than in that of healthy volunteers (35). These authors identified Th17.1 cells, and not Th1 cells, as being the major producers of IFN-γ in the BALF of sarcoidosis patients (35). The proportion of lung Th17.1 cells at the time of diagnosis was higher in sarcoidosis patients who later developed chronic disease than in those who did not, supporting the idea that Th17.1 cells play a pathogenic role in promoting disease progression (20). In contrast, Kaiser et al. reported that a higher percentage of Th17.1 cells was correlated with a disease phenotype with a more favorable prognosis (19). To determine if Th17.1 cells play a pathogenic or protective role in the development of sarcoidosis, further investigation is needed.

The increased frequency of Th17.1 cells in sarcoidosis patients is apparently localized to the involved organs, such as the lung, as reflected by BALF analysis, or the mediastinal lymph nodes (20), but is absent in the blood, indicating that the lung microenvironment is a crucial effect. In our study, we also observed that there was a pronounced proportion of lung Th17.1 cells in the BALF of sarcoidosis patients, but barely detected them in these patients' blood. This imbalanced distribution of Th17.1 cells between peripheral blood and BALF was observed only in sarcoidosis patients and not in patients with IPF, providing valuable information regarding immune cell compartmentalization to the lungs in various ILDs.

Conclusions and future directions

In summary, we found that there was an increased proportion of Tregs in the peripheral blood, and of Th17.1 cells in the BALF, of sarcoidosis patients. An increased proportion of circulating Tregs was associated with disease activity and a fibrotic radiographic phenotype. The imbalanced distribution of Tregs and Th17.1 cells in the peripheral blood and BALF of sarcoidosis patients suggests that the lung microenvironment plays a significant role in the

immunological pathogenesis of sarcoidosis. Functional studies on Tregs and Th17.1 cells are needed so that their exact roles, whether pathogenic or protective, in the immunological processes driving sarcoidosis can be uncovered.

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 Review Committee and the Ethical Review Committee of the China-Japan Friendship Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HZ designed the research route, collected the clinical information and biological samples, conducted the experiments, analyzed the data, and wrote the manuscript. DJ gave suggestions on the research route design and data analysis. LZ, GZ, and BX contributed to screening and enrolling patients and volunteers, data collection, and analysis. YC provided technical direction for experimental methods and revised the manuscript. UC revised the draft critically. HD contributed to the conception and design of the research, interpreted the data and results, and revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by National Key Technologies R&D Program (Number 2021YFC2500700 & 2016YFC0901101).

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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 25 September 2023
DOI 10.3389/fimmu.2023.1267697

OPEN ACCESS

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RECEIVED 26 July 2023
ACCEPTED 04 September 2023
PUBLISHED 25 September 2023

CITATION
Angelats E and Santamaria P (2023) Lineage origin and transcriptional control of autoantigen-specific T-regulatory type 1 cells.
Front. Immunol. 14:1267697.
doi: 10.3389/fimmu.2023.1267697

Lineage origin and transcriptional control of autoantigen-specific T-regulatory type 1 cells

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T Regulatory type-1 (TR1) cells represent an immunosuppressive T cell subset, discovered over 25 years ago, that produces high levels of interleukin-10 (IL-10) but, unlike its FoxP3+ T regulatory (Treg) cell counterpart, does not express FoxP3 or CD25. Experimental evidence generated over the last few years has exposed a promising role for TR1 cells as targets of therapeutic intervention in immune-mediated diseases. The discovery of cell surface markers capable of distinguishing these cells from related T cell types and the application of next generation sequencing techniques to defining their transcriptional make-up have enabled a more accurate description of this T cell population. However, the developmental biology of TR1 cells has long remained elusive, in particular the identity of the cell type(s) giving rise to *bona fide* TR1 cells *in vivo*. Here, we review the fundamental phenotypic, transcriptional and functional properties of this T cell subset, and summarize recent lines of evidence shedding light into its ontogeny.

KEYWORDS

autoimmunity, T-regulatory (Treg) cells, T-regulatory type 1 (TR1) cells, peptide-MHC, nanomedicine, T-follicular helper cells (Tfh)

Introduction

The TR1 cell subset has been implicated in the maintenance of peripheral tolerance against immune-mediated pathologies. TR1-like cells were first documented in severe combined immunodeficiency (SCID) patients that did not develop graft-versus-host disease (GvHD) after receiving HLA-mismatched fetal liver hematopoietic stem cell transplants (1). Subsequent work by Groux et al. using antigen-activated $CD4^+$ T cells cultured in the presence of IL-10 led to the identification of a distinct T cell subset, thereafter named TR1, that could prevent the development of experimental colitis in an IL-10- and transforming growth factor beta (TGFβ)-dependent manner (2).

Unfortunately, the paucity of information on TR1 cell-specific surface markers or transcription factors have hampered the execution of detailed studies on the role and function of this T cell subset in the maintenance or breakdown of self tolerance. The use of relatively non-specific markers of TR1 cell identity, leading to the implication of this subset in various immunological processes (i.e., sometimes relying exclusively on IL-10 expression), has muddied progress in this area. Fortunately, the last decade has witnessed the discovery of phenotypic and molecular features of 'TR1-ness' that have allowed a better definition of TR1-like cells in various experimental settings. These developments, coupled to recent methodological developments in *in vitro* TR1 cell generation (3–7), and the discovery of pharmacological approaches capable of eliciting the formation and expansion of antigen-specific TR1 cells *in vivo* (8), have exposed TR1 cells as attractive targets for therapeutic intervention in immune-mediated diseases.

Despite this progress, significant knowledge gaps remain, including a detailed understanding of the developmental biology processes responsible for the genesis of this T cell subset *in vivo*. The use of novel technologies, including mass cytometry and next-generation-sequencing to address these various gaps are beginning to shed light into these areas of scientific inquiry. In this review, we summarize current knowledge on the phenotypic and molecular hallmarks of TR1 cells and key developmental processes underlying TR1 cell genesis, including recent evidence pointing towards T follicular helper (Tfh) cells as TR1 cell precursors (9, 10).

Phenotype

TR1 cells were initially described as $CD4^+$ T cells producing high levels of IL-10 and IL-5, intermediate levels of TGFβ and INFγ and low levels of IL-4 and IL-2, and were capable of suppressing specific immune responses *in vitro* and *in vivo*, in an IL-10-dependent manner (2). With rapid IL-10 production kinetics, detectable even 4 hours post-activation, and a peak of production at 24h (11), IL-10 became the hallmark cytokine for the TR1 population and, together with the absence of FoxP3 expression, used to identify TR1 cells in early studies. We now know that these criteria are insufficient, given that other $CD4^+$ T cell types such as Th1 (12, 13) or Th2 (14) can produce IL-10 and acquire immunoregulatory properties; such cells do not belong to the TR1 subset. For example, Lönnberg et al. claimed a Th1 origin for TR1 cells in a chronic *Plasmodium* infection model, solely on the basis of presence of IL-10-expressing cells within the infection-induced Th1 pool, and on the assumption that TR1 cells are simply IL-10/IFNγ-co-expressing cells (15). In fact, further transcriptomic analyses of the $IL\text{-}10^+$ and $IL\text{-}10^-$ Th1 cells of these mice revealed the presence of only two differentially expressed genes between these subsets (*Trib2* and *BC017643*). In another study, also in a chronic *Plasmodium* infection model, Soon et al. reported a similar outcome; 34% of Th1 lineage cells co-expressed *Ifng* and *Il10* (16). It is thus likely that, based on the evidence provided, the $IL\text{-}10^+$ cells that arose in these mice were IL-10-expressing Th1 cells, rather than true TR1 cells. This indicates that the assignment of TR1ness cannot merely rely on IL-10 expression.

In light of these challenges, hampering progress in defining the significance of the TR1 cell subset in both physiology and pathology, extensive efforts were made to better describe the molecular hallmarks of the TR1 subset [Reviewed in (17)]. Notwithstanding the fact that markers strictly unique to TR1 cells remain elusive, recent advances have made it possible to more accurately identify such cells in biological samples.

Gagliani and colleagues identified co-expression of CD49b and Lymphocyte-activation gene 3 (LAG-3) as surface markers for both human and murine IL-10-producing TR1-like cell populations (18). Subsequent studies indicated that a significant fraction of these IL-10-producing $CD49b^+LAG\text{-}3^+$ T cells are co-inhibitory receptor-rich, expressing Programmed cell death-1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T-cell immunoglobulin and mucin-domain containing protein-3 (TIM-3), and Cytotoxic T-Lymphocyte antigen 4 (CTLA-4), and co-express the co-stimulatory molecule ICOS (Inducible Costimulator) and the chemokine receptor CCR5, among other molecules (7). In agreement with these observations, intestinal TR1-like cells expressing PD-1 and CCR5 were found to co-express CD49b and LAG-3 by others (19), thus supporting the use of such markers for TR1-like cell identification. Thus, as proposed elsewhere (20), TR1 cell annotation should meet the following four criteria: 1) high IL-10 production competency (co-expression of other cytokines in variable amounts depending of environmental cues is possible); 2) immunoregulatory activity; 3) absence of constitutive FoxP3 expression (expression of FoxP3 upon activation, particularly in human TR1 cells, is not an exclusion criterium); and 4) co-expression of CD49b and LAG-3 in the presence of other co-inhibitory receptors such as PD-1, TIGIT, TIM-3 or CTLA-4 among others.

Indeed, the profoundly immunoregulatory antigen-specific TR1-like cells that arise *in vivo* in response to systemic delivery of nanoparticles (NPs) coated with mono-specific disease-relevant peptide-Major Histocompatibility Complex class II (pMHCII) molecules (8, 21–23) lack FoxP3 expression and upregulate many of the markers mentioned above, including CD49b and the co-inhibitory receptors LAG-3, PD-1, TIGIT and CTLA-4, the co-stimulator ICOS, the cytokines IL-10, IL-21 and IFNγ and the chemokine receptors CCR5 and CXCR3 (9). We have shown that administration of these compounds can lead to the resolution of inflammation in various organ-specific autoimmune disease models in a disease-specific manner without impairing normal immune responses (8, 21, 22). Adoptive transfer experiments demonstrated that the cognate ($pMHCII\ tetramer^+$) T cells arising in these mice in response to therapy were largely, albeit not exclusively, responsible for the therapeutic properties of these compounds (TR1 cell-induced B-regulatory cells also contributed to disease suppression) (8, 22).

Mechanisms of action

TR1 cells need to be activated to immunoregulate. Upon recognition of their cognate pMHCII complexes on co-stimulation-competent APCs, TR1 cells become productively

activated. By actively inhibiting the antigen-presentation and pro-inflammatory properties of these APCs (in addition to direct effects on other target cell types, see below), TR1 cells can suppress both cognate and non-cognate effector T cell activation (a process referred to as 'bystander immunoregulation'). This biological activity involves the deployment of several mechanisms (Figure 1).

Production of immunoregulatory cytokines

As noted above, productive activation of TR1 cells leads to rapid and robust production of IL-10, which can suppress the function of different immune cell subsets, such as T cells, APCs and B cells. IL-10 can inhibit the proliferation of, and downregulate the production of effector cytokines by, effector T cells (24), and can induce an anergic state in T cells in a STAT3-dependent manner (25). Likewise, IL-10 can inhibit the production of pro-inflammatory mediators by professional APCs, and downregulate the expression of MHC class II molecules and co-stimulatory molecules on their surface (26). It can also promote the upregulation of the immunoglobulin-like transcripts 3 and 4 (ITL3 and 4) and HLA-G, which have been implicated in the generation of tolerogenic dendritic cells (DCs) (27). On B cells, IL-10 promotes proliferation, expression of MHC class II molecules and isotype switching to IgG4 (28).

TGFβ has also been implicated in TR1-mediated immunoregulation. This cytokine suppresses T cell proliferation via various mechanisms, such as by inducing the downregulation of cyclins and IL-2 and the upregulation of cyclin-dependent-kinases (CDKs) (29–31). TGF-β can also suppress the formation of effector CD4+ or CD8+ T cells by inhibiting the expression of the master Th1 and Th2 cell transcriptional regulators (T-bet and GATA-3, respectively) (32, 33) or the IL-12Rβ2 chain (34).

The contribution and importance of both cytokines, IL-10 and TGFβ, to the immunosuppressive activity of TR1 cells is exemplified by the fact that, blockade of these cytokines inhibits TR1 cell-mediated immunoregulation in various experimental settings, including pMHCII-NP-treated animals (2, 8).

An additional cytokine that has been implicated in TR1-mediated immunoregulation is IL-21. Whereas IL-10 is directly responsible for most of the regulatory properties of pMHCII-NP-induced TR1 cells, IL-21 contributes to sustaining IL-10 expression in TR1 cells and is directly responsible for TR1-induced Breg cell formation (8).

Engagement of co-inhibitory and co-stimulatory molecules

Engagement of the TR1 cells' co-inhibitory (i.e., LAG-3, CTLA-4, TIGIT or PD-1) and co-stimulatory receptors (i.e., ICOS) by the corresponding ligands on target cells, such as APCs, is also thought to play a role in their immunoregulatory activity. Indeed, all these molecules are upregulated on the TR1-like cells induced by

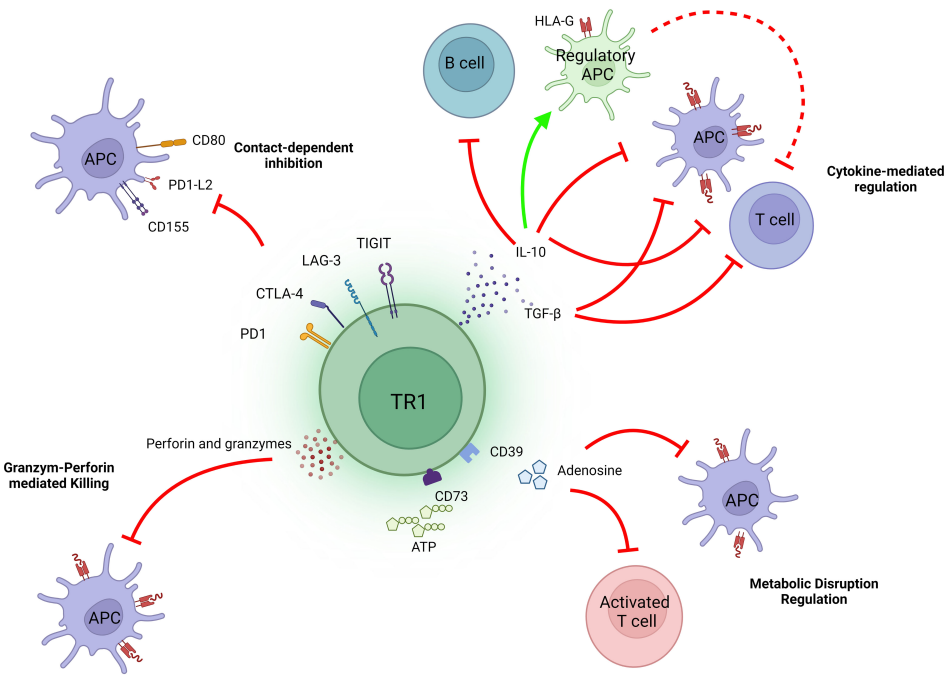

FIGURE 1
Mechanisms of action of TR1 cells. The immunosuppressive activity of TR1 cells is largely, albeit not exclusively, mediated through the release of IL-10 and TGFβ. Both cytokines can directly inhibit effector T cells and APCs, the latter also having a large indirect impact on effector T cell function. Additionally, IL-10 can imprint regulatory properties in APCs, such as by promoting the upregulation of tolerogenic molecules like HLA-G. The co-inibitory molecules expressed on TR1 cells, including CTLA-4, PD-1, TIGIT and LAG-3, can also result in contact-dependent inhibition of APCs and consequently, inhibit APC-induced T cell activation. Upon recognition of cognate pMHCII complexes on APCs, TR1 cells can also kill these cells via perforin and granzymes. In addition, these TR1 cells can inhibit T cell function by producing adenosine.

pMHCII-NP therapy (9). Whereas engagement of co-inhibitory receptor ligands on APCs by the TR1 cells' co-inhibitory receptors may contribute to the suppression of the APC's function, engagement of co-stimulatory receptor ligands (along with cognate pMHCII) elicits the productive activation of the TR1 cells, leading to secretion of the TR1 cells' immunoregulatory cytokines. In turn, these molecules have immunoregulatory effects on APCs and other downstream cellular targets.

LAG-3 negatively regulates T cell activity. Structurally similar to the CD4 co-receptor, LAG-3 recognizes MHCII molecules with higher affinity than CD4 (35). Recent evidence has shown that engagement of LAG-3 by stable pMHCII complexes transduces intracellular inhibitory signals to the T cell, without interfering with the recognition of these complexes by the T cells' TCR or CD4 molecules (36). Although such a mechanism helps understand how LAG-3 upregulation by an effector T cell might suppress its activation, a T cell-intrinsic inhibitory role for LAG-3 on regulatory T cell activity/function (37) seems counter-intuitive, as it would suppress the Treg cell, suggesting the existence of alternative mechanisms. One possibility is that the interaction between LAG-3 on Treg cells and pMHCII on APCs exclusively results in suppression of the latter, perhaps by failing to transduce intracellular inhibitory signaling into the former. The finding that such interaction results in the inhibition of dendritic cell (DC) activation (38), supports this possibility.

CTLA-4, a member of the CD28 family, binds to the co-stimulatory ligands CD80/86 with higher affinity than CD28, inhibiting the activation of the latter. In addition, the CTLA-4–CD80/86 interaction promotes the dephosphorylation of CD3 and CD28 signalling intermediates through the Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2), promoting T cell inhibition (39). Although CTLA-4 is dispensable for peripheral Treg cell expansion, it is necessary for immunoregulatory activity (40). There is also evidence indicating that CTLA-4 (along with PD-1) plays an active role in the regulatory activity of TR1 cells (41). It therefore seems likely that the role of CTLA-4 expression on TR1/Treg cells is different than that of CTLA-4 upregulation by effector T cells, although this remains to be determined (42).

The PD-1 receptor binds PD-L1 or PD-L2, expressed predominantly on APCs. Upon interaction with PD-L2, PD-1 on effector T cells recruits SHP-1 and SHP-2 phosphatases, which in turn reduce T cell activation and induce Treg cell differentiation (43). On DCs, the PD1–PD-L2 interaction inhibits the expression of molecules associated with DC maturation such as CD80, CD86 or CD40 and induces IL-10 expression, thus promoting the induction of an immunosuppressive DC phenotype (44).

Although the intracellular domain of TIGIT contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) capable of recruiting SHIP-1 and thus suppress T-cell (and NK cell) activation (45), binding of TIGIT to CD155 or CD112 on APCs (with high and low affinity, respectively), inhibits the engagement of the CD226 co-stimulator on T cells (46). In addition, this interaction induces a tolerogenic phenotype in DCs, by promoting IL-10 and suppressing IL-12 production (47).

Binding of the co-stimulator ICOS on TR1 cells to its ligand, ICOS-L, on B cells, DCs or macrophages (in the context of a cognate TCR-pMHCII interaction) promotes TR1 cell activation, leading to secretion of regulatory cytokines such IL-10 (48–50).

Extracellular generation of adenosine

TR1 cells, including those arising in response to pMHCII-NP therapy (9) express ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73), which hydrolyze extracellular adenosine triphosphate (ATP) released during T cell activation (51). This leads to the generation of adenosine (52, 53), which binds to the G protein-coupled adenosine receptor A2 ($A_{2A}R$). This interaction elicits a signalling cascade that suppresses effector T cell proliferation and cytokine production (54). On APCs, binding of adenosine to $A_{2A}R$ promotes IL-10 expression and inhibits both their maturation and their ability to secrete pro-inflammatory cytokines (55).

Granzyme and perforin-mediated killing

Another mechanism that TR1 cells may use to regulate T cell activity involves the killing of cognate APCs (i.e., expressing the TR1 cells' target pMHCII) via granzyme A and B and perforin (56, 57). By killing APCs, TR1 cells can thus suppres the activation of other T cell specificities and promote bystander immunoregulation. However, our work in mice treated with autoimmune disease-relevant pMHCII-NPs suggest that this mechanism is not always at play. For example, the antigen-specific TR1 cells emerging in these mice upon pMHCII-NP therapy did not significantly upregulate perforin and did not kill antigen-expressing or peptide-pulsed APCs (B cells or DCs) *in vivo* (8), unlike the case for the regulatory CD8+ T cells arising in pMHCI-NP-treated animals (58).

Molecular and transcriptional regulation of TR1 cell specification

Extensive efforts over the last two decades have sought to define the molecular and transcriptional mechanisms orchestrating TR1 formation. Unlike the case for the FoxP3+ Treg cell subset, where expression of FoxP3 is central to the acquisition of its immunoregulatory properties, there is no known unique master transcriptional regulator of TR1 cell development. Notwithstanding this limitation, experimental evidence has implicated a number of cytokines, kinases and transcription factors in the generation of TR1 cells *in vitro*. Although TCR engagement in the presence of IL-10 appears to play a major role, other signals are also required.

There is evidence suggesting that superantigens (59, 60) and pMHCII multimers can induce the expression of IL-10 in CD4+ T cells (61–63). It has also been shown that high-avidity TCR–pMHCII interactions favour the production of IL-10 by T cells (64), affecting both the number of cells expressing IL-10 and the immunoregulatory properties of such cells (65). Molecularly, TCR activation leads to the engagement of intracellular signalling

pathways that eventually activate the interferon regulatory factor 4 (IRF4) transcription factor via Ras or the inducible tyrosine kinase (ITK) kinases. IRF4 has been shown to promote *Il10* gene expression in different CD4+ T cell types, including Th2, Th1 (66), Tfh cells (67) and Tregs (68). Indeed, it has been reported that IRF4 contributes to the development of an IL-10-producing CD4+ T cell that co-expresses LAG-3 and CD49b (69). As noted below, IRF4 is absolutely required for pMHCII-NP-induced TR1 cell formation, albeit through a different mechanism (i.e., it is dissociated from its *Il10* transactivating function) (9). The transcription factor Eomes, which can promote *Il10* expression in T-bet-expressing cells (70), and the Th17 transcription factor Rorα, which can transactivate the *Il10* gene (71), might also be implicated in TR1 formation. However, pMHCII-NP-induced TR1 cells do not upregulate Eomes or Rorα, suggesting that neither of these transcription factors are required for TR1 cell specification.

Although productive TCR ligation is required for TR1 activation, TR1 cell genesis requires additional cues. Early studies by Groux and colleagues using both human and murine CD4+ T cells cultured in presence of IL-10 indicated that these culture conditions promoted the development of an anergic T cell population that included TR1-like cells (2), highlighting a prominent role for this cytokine in TR1 cell generation, at least *in vitro*. It was subsequently proposed that the IL-10 that contributes to TR1 cell generation *in vivo* derives from a tolerogenic DC population (72, 73). Indeed, a human DC population expressing high amounts of IL-10 has been identified (27). This DC population, named DC-10, can induce TR1 cells *ex vivo* with increased efficacy, as compared to other experimental approaches, and such cells have been used to generate and expand TR1 cells for use in clinical trials [Reviewed in (5)].

IL-27 has also been implicated in the generation of murine TR1 cells. IL-27 is an IL-12 family cytokine that is produced by activated APCs (74) and can induce IL-10 expression in murine T cells (75–77), especially in the presence of TGFβ, with which it synergizes (78). Binding of IL-27 to the IL-27 receptor (IL-27R) activates the STAT1 and STAT3 signalling pathways and promotes the expression of the transcription factors c-Maf and AhR, which cooperatively promote *Il10* and *Il21* expression (78, 79). STAT3-induced *Il10* expression also involves the upregulation of *Egr2* (encoding the Early Growth Response 2 transcription factor (EGR-2)) and EGR-2's downstream target *Prdm1* (encoding the zinc finger-containing transcription factor Blimp-1) (80). Although Blimp-1 has been primarily implicated in plasma cell differentiation (81), it has also been shown to regulate *Il10* gene expression in T cells (82, 83). However, and notwithstanding the fact that the generation of terminally differentiated TR1 cells in response to pMHCII-NPs requires Blimp-1, this role is dissociated from Blimp-1's *Il10* transactivating function (9) (see below). In fact, there is evidence suggesting that IL-27-induced TR1 cell formation does not require IL-27-induced IL-10 (84) and that IL-27 contributes to TR1 cell formation by inducing changes in chromatin accessibility via IRF1 and BAFT (85). Although IL-27 can induce the formation of IL-10-expressing T cells from naïve human CD4+ precursors (86), it remains to be determined whether these cells are *bona fide* TR1 cells.

Despite all these observations, largely if not exclusively generated *in vitro*, our *in vivo* work has demonstrated that IL-27 is not required for pMHCII-NP-induction of TR1-like cells (8). We have proposed that IL-27 and pMHCII-NPs lie upstream and downstream of the TR1 precursors (Tfh cells, see further below); whereas IL-27 would elicit both Tfh and TR1 cell formation from naïve precursors, pMHCII-NPs would just be able to promote the conversion of Tfh cells into TR1-like cells (9).

IL-21 is another cytokine that has been implicated in TR1 cell genesis. This cytokine, produced by antigen-stimulated CD4+ T cells and NKT cells, signals via the IL-21R, composed of the IL-21Rα chain and the common receptor γ_c chain (87), leading to activation of the STAT3 signalling pathway. The transcription factor c-Maf, upregulated by IL-27 among other stimuli, promotes IL-21 expression in TR1 cells (79, 88). In turn, IL-21 promotes the expression of *Il10* and *cMaf* expression in an autocrine manner (79, 89).

In addition to the molecules discussed above, other cytokines and transcription factors have been reported to contribute to IL-10 production by T cells and, consequently, may play a role in TR1 cell specification. For instance, IL-6, which signals through STAT1 and STAT3, can upregulate the transcription factors c-Maf, IRF4 or AhR (90), which are known to participate in *Il21 and/or l10* expression in different T cell types (66–69, 78, 79). In fact, IL-6, together with TGFβ, can induce IL-10 production in Th17 cells (91, 92). Type-1 Interferons have also been reported to promote IL-10 expression in CD4+ T cells (93–95) or TR1 cell development in anti-CD3 mAb/IL-10-treated mice (96).

The co-stimulator ICOS may also play an important role in TR1 cell specification, homeostasis or function, perhaps by promoting *cMaf* and *Il10/Il21* expression (88, 97).

In summary, research to date has provided valuable information regarding the transcriptional control of *Il10*, encoding the hallmark TR1 cytokine, but has not yet been able to define the key transcription factors, co-stimulators and cytokines that control TR1 cell development from their precursors. As summarized below, the recent identification of Tfh cells as precursors of TR1 cells *in vivo*, coupled with definition of the transcriptional changes that underlie this transdifferentiation process, offer a unique opportunity to carefully map the molecular events responsible for TR1 cell formation.

Challenges hampering studies on the developmental biology of TR1 cells

Given the challenges associated with the lack of TR1 cell-specific markers and our inability to reliably identify this T cell subset *in vivo* until recently, it is unclear whether the TR1-like cells that have been described to arise *in vitro* and/or *in vivo* in response to various cues do so from a single or various precursors [reviewed in (98, 99)].

As noted above, both human and mouse T cells can be differentiated into TR1-like cells *in vitro*. *In vitro*-activated naïve T cells from either species can give rise to anergized IL-10-

producing CD4+ T cells when cultured in the presence of exogenous IL-10 (2), DC-10 cells (100) or IL-27 (79, 101).

Several other lines of evidence have suggested that TR1 cells arise from memory T cell precursors. Repetitive administration of anti-CD3 monoclonal antibodies (mAb) to mice can induce TR1-like cell formation *in vivo* (102). *In vitro* stimulation of memory-like $CD4^{+}CD44^{high}FoxP3^{-}$ T cells in the absence of polarizing cytokines can also elicit TR1-like cell specification (103). Likewise, extracellular matrix components have been reported to guide the formation of TR1-like cells from human memory $CD4^{+}$ T cells *in vitro* (104), and others have shown that the precursors of TR1 cells are contained within the memory $CD4^{+}$ T cell pool, in both humans and mice (105).

Th1 and Th2 cells have also been proposed as a source of TR1-like cells. *In vitro*, Th1 cells can be induced to express IL-10 when stimulated in the presence of CXCL12 (106), but such cells might have just been IL-10-producing Th1 cells rather than full-fledged TR1 cells. As noted earlier in this review, it has been suggested that chronic infection of mice with *Plasmodium* can trigger the differentiation of Th1 cells into TR1-like cells (15, 16), but the reported TR1-like cells did not appear to be *bona fide* TR1 cells. It has also been suggested that allergen-specific Th2 cells can be re-programmed into a TR1-like phenotype *in vitro* (107).

Intestinal Th17 cells can also give rise to anti-colitogenic TR1-like cells in response to anti-CD3 mAb treatment (108). Furthermore, IL-27 and IL-23 promote the up-regulation of Blimp-1 and can elicit the expression of a TR1-like phenotype in Th17 cells (109).

Collectively, the above observations suggest that TR1-like cells might arise from various T cell precursor types, but many of these studies did not use stringent criteria for definition of the TR1 cell state, or did not involve detailed transcriptional studies of the T cell pools used for experimentation or of their progeny; and when such studies were done/reported, the resulting T cell pools were transcriptionally heterogeneous. As a result, it is not possible to unambiguously assign or exclude a specific cell type as a TR1 cell precursor on the basis of these studies.

Tfh cells as a source of TR1 cells

We have taken advantage of the large pools of antigen-specific TR1 cells that arise *in vivo* in various animal models of autoimmunity upon systemic delivery of nanoparticles (NPs) coated with disease-relevant pMHCII molecules (8, 21–23), to carefully map the transcriptional events leading to TR1 cell formation *in vivo* (9, 10) (Figure 2). Early work established that pMHCII-NPs functioned by re-programming cognate antigen-experienced (i.e. memory) $CD4^{+}$ T-cells of unknown identity (excluding a role for naïve T-cells) (8, 23).

The robust and prolongued TCR signaling events that result from sustained assembly of TCR microclusters by pMHCII-NPs on autoantigen-experienced T cells leads to the expression of known TR1-like cell markers, including IL-10, IL-21, c-Maf, LAG-3, CD49b, CTLA-4, PD-1, TIGIT, CCR5, CXCR3, ICOS and OX-40, among others, in a manner that does not require APCs or IL-27 (9).

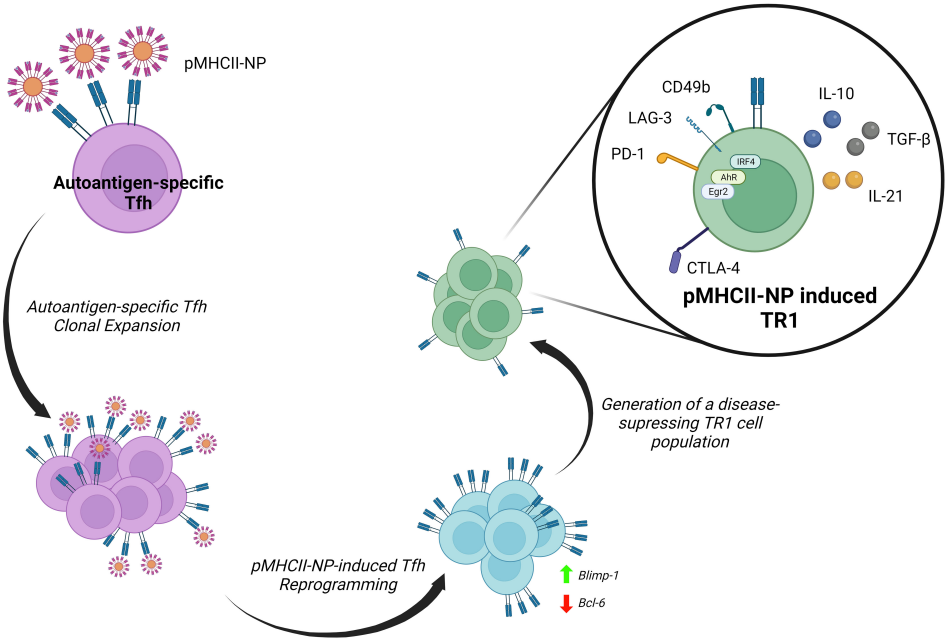

FIGURE 2
Reprogramming of Tfh cells into TR1 progeny by pMHCII-NPs. Upon binding to cognate TCRs, autoimmune disease relevant pMHCII-NPs selectively trigger the clonal expansion of Tfh cells and their immediate transdifferentiation into transitional TR1-like cells and terminally differentiated TR1 cells. pMHCII-NP encounters trigger the downregulation of master transcriptional regulators of Tfh cells, such as Bcl-6, and the upregulation of Blimp-1. Blimp-1 expression is a *sine-qua-non* requirement for the conversion of transitional TR1-like cells into the terminally differentiated, immunoregulatory TR1 cell subset (9).

In addition to *Maf*, these TR1 cells upregulate the transcription factor coding genes *Ahr, Egr2, Irf4, Nfil3, Prdm1 and Tbx21* (9), all involved in IL-10 expression (79, 110). In addition, these TR1 cells upregulate three other transcription factors that have been previously implicated in the development, maintenance or function of IL-10-expressing Treg cells (*Bhlhe40*, *Runx2* and *Vdr*) (9). Whereas the IL-10 produced by these antigen-specific TR1 cells is the direct mediator of some of their immunoregulatory properties, IL-21 contributes to the homeostatic regulation of this T-cell subset and plays a critical role in TR1-induced Breg cell formation (8). This ability of pMHCII-NPs to elicit the formation of large pools of antigen-specific TR1-like cells afforded us a unique opportunity to explore their developmental biology. This work has demonstrated that pMHCII-NP-induced TR1 cells derive from cognate Tfh cells and do so in a Blimp-1-dependent manner (9, 10) (Figure 2).

Initial work indicated that the cognate TR1-like cell pools arising in response to pMHCII-NPs expressed a transcriptional program that shared significant features with Tfh cells, raising the possibility that the latter might function as a source of the former. Subsequent single cell RNA sequencing (scRNAseq) and mass cytometry studies demonstrated that these antigen-specific TR1-like cell pools harboured a cognate Tfh-like cell subcluster, in addition to its TR1-like cell countepart. Importantly, studies of the TCR repertoires of these two cell sub-clusters indicated that they consistently harbored identical clonotypes, thus demonstrating that they were developmentally related (9, 10). This was substantiated with the use of different pMHCII-NP types in different genetic backgrounds and models of autoimmunity (9, 10).

This was further documented by demonstrating that pMHCII-NPs could elicit cognate TR1 cell formation in immunocompromised hosts transfused with purified Tfh cells, and that these compounds lacked pharmacodynamic activity in mice unable to generate Tfh cells (9). Most importantly, T cell-specific deletion of *Prdm1* (encoding Blimp-1) revealed that the Tfh-to-TR1 cell conversion evolves through a transitional (TR1-like) subset, and that expression of this transcription factor in these transitional T cells is a *sine qua non* requirement for full-fledged acquisition of the TR1 transcriptional profile and regulatory function (9). Thus, while specific deletion of *Bcl6* or *Irf4* in T-cells blunted pMHCII-NP-induced cognate CD4+ T-cell expansion and downstream TR1 generation, deletion of *Prdm1* enabled the former but completely abrogated the latter (9).

It could be argued that pMHCII-NP-induced TR1 cells are T-follicular regulatory (TFR) cells, which negatively regulate the germinal center (GC) reaction (111). However, unlike TR1 cells, TFR cells express CXCR5 (but not CCR5), Bcl-6, FoxP3 and CD25, and arise from natural FoxP3+ Treg cell precursors in a Blimp-1-independent manner (9).

Together, the data summarized above conclusively demonstrate that murine TR1 cells can arise from Tfh cells in a Blimp-1-dependent manner. Interestingly, a pool of thymus-derived self-reactive CD4+ T cells that adopt numerous hallmarks of Tfh cell identity in the periphery has been recently discovered (112). This finding raises the possibility that these cells might function as a source of a negative feedback regulatory loop (i.e., formation of autoreactive TR1 cells) to suppress autoimmunity.

Concluding statement

Since the discovery of TR1-like cells more than 25 years ago, the last decade has witnessed steady improvements in our ability to identify and phenotype this previously enigmatic CD4+ T cell subset. While knowledge gaps persist, we have gained detailed new insights into these cells' transcriptional make-up, mechanisms of action and lineage origin. Further research into the different topics reviewed in this article, as well as other aspects of the TR1 cell biology will undoubtedly help in the translational application of TR1 cells as a therapeutic approach for immune-mediated diseases.

Author contributions

PS: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review and editing. EA: Investigation, Writing – original draft.

Funding

This work was supported by the Canadian Institutes of Health Research (CIHR; FDN-353029, PJT-479040, PJT-479038, FRN-168480 (with JDRF), and DT4-179512), Genome Alberta and Genome Canada (GAPP program), the Praespero Foundation, the Alberta Diabetes Foundation, Ministerio de Ciencia e Innovación of Spain (MICIN; PID2021-125493OB-I00), the ISCIII and FEDER (PIE14/00027, PI15/0797), MINECO, Generalitat de Catalunya (SGR and CERCA Programmes), Generalitat de Catalunya (SGR and CERCA Programmes), and and Red Española de Supercomputación (RES, providing CSUC resources).

Acknowledgments

We thank the members of our laboratory for their technical and conceptual contributions. Cartoons for Figures 1 and 2 were created with BioRender.

Conflict of interest

PS is founder, scientific officer and stock holder of Parvus Therapeutics. He is inventor on patents on pMHC-based nanomedicines and receives funding from the company.

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 25 September 2023
DOI 10.3389/fimmu.2023.1253649

OPEN ACCESS

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RECEIVED 05 July 2023
ACCEPTED 08 September 2023
PUBLISHED 25 September 2023

CITATION
Yilmaz K, Haeberle S, Kim YO, Fritzler MJ, Weng SY, Goeppert B, Raker VK, Steinbrink K, Schuppan D, Enk A and Hadaschik EN (2023) Regulatory T-cell deficiency leads to features of autoimmune liver disease overlap syndrome in scurfy mice.
Front. Immunol. 14:1253649.
doi: 10.3389/fimmu.2023.1253649

Regulatory T-cell deficiency leads to features of autoimmune liver disease overlap syndrome in scurfy mice

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Introduction: Scurfy mice have a complete deficiency of functional regulatory T cells (Treg) due to a frameshift mutation in the *Foxp3* gene. The impaired immune homeostasis results in a lethal lymphoproliferative disorder affecting multiple organs, including the liver. The autoimmune pathology in scurfy mice is in part accompanied by autoantibodies such as antinuclear antibodies (ANA). ANA are serological hallmarks of several autoimmune disorders including autoimmune liver diseases (AILD). However, the underlying pathogenesis and the role of Treg in AILD remain to be elucidated. The present study therefore aimed to characterize the liver disease in scurfy mice.

Methods: Sera from scurfy mice were screened for ANA by indirect immunofluorescence assay (IFA) and tested for a wide range of AILD-associated autoantibodies by enzyme-linked immunosorbent assay, line immunoassay, and addressable laser bead immunoassay. $CD4^+$ T cells of scurfy mice were transferred into T cell-deficient B6/nude mice. Monoclonal autoantibodies from scurfy mice and recipient B6/nude mice were tested for ANA by IFA. Liver tissue of scurfy mice was analyzed by conventional histology. Collagen deposition in scurfy liver was quantified via hepatic hydroxyproline content. Real-time quantitative PCR was used to determine fibrosis-related hepatic gene expression. Hepatic immune cells were differentiated by flow cytometry.

Results: All scurfy mice produced ANA. AILD-associated autoantibodies, predominantly antimitochondrial antibodies, were detected at significantly higher levels in scurfy sera. $CD4^+$ T cells from scurfy mice were sufficient to induce anti-dsDNA autoantibodies and ANA with an AILD-related nuclear envelope staining pattern. Liver histology revealed portal inflammation with

bile duct damage and proliferation, as in primary biliary cholangitis (PBC), and interface hepatitis with portal-parenchymal necroinflammation, as found in autoimmune hepatitis (AIH). In scurfy liver, TNFα and fibrosis-related transcripts including *Col1a1, Timp1, Acta2, Mmp2*, and *Mmp9* were upregulated. The level of proinflammatory monocytic macrophages (Ly-6C^{hi}) was increased, while M2-type macrophages (CD206^{+}) were downregulated compared to wildtype controls. Despite severe hepatic inflammation, fibrosis did not develop within 25 days, which is close to the lifespan of scurfy mice.

Discussion: Our findings suggest that Treg-deficient scurfy mice spontaneously develop clinical, serological, and immunopathological characteristics of AILD with overlapping features of PBC and AIH.

KEYWORDS

regulatory T cells, Treg, scurfy mice, autoimmune liver disease, overlap syndrome, primary biliary cholangitis, autoimmune hepatitis

1 Introduction

Regulatory T cells (Treg) represent a distinct subset of CD4^{+} lymphocytes which play a pivotal role in the maintenance of peripheral tolerance by actively preventing autoimmunity (1). The development and suppressive functions of Treg are essentially regulated by constitutive expression of the transcription factor Forkhead Box 3 (FoxP3) (2). As such, a disruption of the encoding gene gives rise to rampant expansion of autoreactive CD4^{+} T cells, which then infiltrate several organs, exacerbate and perpetuate tissue insult by recruiting other inflammatory cells. The consequence is a lethal systemic autoimmune disorder with multi-organ failure, as manifested by immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans and genetically equivalent *Foxp3* mutant scurfy mice (3, 4). Due to a complete deficiency in functional Treg, hemizygous male scurfy mice spontaneously develop a lymphoproliferative disease with systemic inflammation, particularly involving the skin, kidneys, lung, and the liver, and resulting in death within four weeks of age (5, 6). The loss of immune homeostasis does not only rely on T-cell dependent mechanisms but is also orchestrated by B cells via production of autoantibodies, including antinuclear antibodies (ANA) (7–11).

ANA represent serological biomarkers of a variety of systemic autoimmune disorders such as connective tissue disease (CTD) and autoimmune liver diseases (AILD) (12). Based on clinical, histopathological, and serological findings, three major immune-mediated liver diseases can be distinguished, i.e., autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). Although most cases match criteria of one of these entities, features of multiple categories may rarely occur concomitantly within the spectrum of AILD, a clinical phenotype referred to as "overlap syndrome" (13). Despite well-defined diagnostic parameters, the current therapeutic armamentarium is predominantly limited to non-specific immunosuppression and/or anti-cholestatic agents. Hence, if insufficiently treated, chronic inflammation and protracted repair mechanisms in AILD can lead to liver fibrosis and ultimately to cirrhosis, the strongest predisposing factor for hepatocellular carcinoma (14, 15). Of note, liver dysfunction was also reported in patients with IPEX syndrome, which is mostly fatal within the first two years of life (16).

The precise pathogenesis underlying AILD and the specific role of Treg in these diseases are still elusive. Thus, the present study sought to characterize the hepatic disease spontaneously evolving in scurfy mice. In this context, we screened sera of scurfy mice for the existence of ANA by an indirect immunofluorescence assay (IFA), analyzed the staining patterns, and tested for autoantibodies against targets specific to or associated with AILD, i.e. antimitochondrial antibodies with three major epitopes (AMA-MIT3), valosin-containing protein/p97 (VCP), glycoprotein-210 (gp210), Kelch-like protein (KL), hexokinase (HK), lamin B1, liver cytosol type 1 (LC1), soluble liver antigen (SLA), liver kidney microsome (LKM), soluble protein 100 kDa (sp100), early endosomal antigen 1 (EEA1), Ge-1, glycine-trytophan protein of 182 kDa (GW-182), and argonaute protein (Ago2). We further examined histopathological alterations, fibrosis-related transcripts, and the cellular components of the inflammatory infiltrates in scurfy liver. Our findings indicate that Treg-deficient scurfy mice harbor clinical and serological features of AILD with overlapping characteristics of AIH and PBC.

2 Materials and methods

2.1 Mice

Female heterozygous *B6.Cg-Foxp3sf/J* (Scurfy) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred to *C57BL/*

6 wild-type (WT) male mice to generate hemizygous male *B6.Cg-Foxp3sf/Y* (scurfy) offspring. *B6.Cg-Foxn1nu/J* (B6/nude) mice were acquired from Jackson Laboratories. All mice were maintained under specific pathogen-free conditions at the central animal facility of the Interfaculty Biomedical Facility, University of Heidelberg, Germany. Animal work was performed in line with the animal protocols (35-9185.81/G-195/11, T13/16, and T58/16), approved by the animal care committee (Regierungspräsidium Karlsruhe).

2.2 Screening for antinuclear antibodies

Serum samples taken from scurfy and WT mice at day 21 of life were assessed for the presence of ANA by an IFA, as previously described (10). Briefly, sera were diluted in PBS with 0.2% Tween 20 (Roth, Karlsruhe, Germany) and were added to slides precoated with human epithelial cells (HEp-20-10) and primate liver tissue (Euroimmun, Lübeck, Germany). Goat anti-mouse IgG Alexa Fluor 488 (dilution 1:500, Invitrogen, Carlsbad, CA, USA) served as secondary antibody. For clarity, although some of the IFA patterns were clearly cytoplasmic, here we collectively refer to both cytoplasmic and nuclear patterns as ANA. IFA images were generated by a fluorescence microscope (Zeiss Axioscop 40, Carl Zeiss, Göttingen, Germany). Semiquantitative analysis was performed in accordance with the manufacturer's recommendations. ANA titers ≥ 1:100 were considered positive. Morphological fluorescence patterns were classified and shown as designated from anti-cellular (AC) 0 (negative) to AC-29, according to the recently updated International Consensus on ANA Patterns (ICAP) (17, 18).

2.3 Detection of antigenic targets of ANA

A group of AILD-related autoantibodies (AMA-MIT3 (against PDC-E2, BCOADC-E2, and OGDC-E2), LKM, sp100, gp210, SLA, LC-1) were identified by Euroimmun Line Immunoassay (LIA; Euroimmun, Lübeck, Germany), as previously reported (19). Antibodies directed to HK and KL were measured by QUANTA Lite enzyme-linked immunosorbent assay (ELISA) (Inova Diagnostics Inc., San Diego, CA, USA) (20). Autoantibodies to GW182, Ago2, Ge-1, EEA1, VCP, and lamin B1 were detected using a laboratory developed multiplexed addressable laser bead immunoassay (ALBIA), as previously described (10, 19, 20). Briefly, 20 microliters (µls) of suspended beads bearing the covalently coupled antigen analyte, 25 µl of sample diluent (Inova Diagnostics Inc., San Diego, CA, USA) and 5 µl of diluted mouse serum were added into the wells of 96-well plate. The plate was incubated with agitation at 600 rpm for 30 min at room temperature (RT), followed by incubation in goat anti-mouse IgG phycoerythrin conjugated secondary antibody (0.5 µg/ml, Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) for 30 min and 600 rpm in the dark. Plates were analyzed by using a Luminex-100 plate reader (Luminex Corp., Austin, TX, USA). Cutoff levels were determined on positive and negative controls in each run and were set at three standard deviations (SD) above the mean for WT mice.

2.4 Hybridoma generation

Prior to the fusion, six scurfy mice were selected based on the presence of ANA by IFA. Total lymph node and splenic cells were pooled and fused with the murine myeloma cell line Sp2/0 (ATCC, Manassas, VA, USA), according to the standard protocol (21). Fused cells were selected by using hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine-thymidin (HT) medium (Sigma-Aldrich, St. Louis, MO, USA), and supernatants were screened for ANA on HEp-20-10 and primate liver tissue by IFA. ANA positive clones were chosen and grown in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) and subcloned repeatedly to assure monoclonality.

2.5 Passive transfer of $CD4^+$ T cells into *nu/nu* mice

$CD4^+$ T cells were isolated from lymph nodes and spleens of scurfy mice and WT controls through magnetic activated cell sorting with CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells (2×10^6; purity >95%) in 100 µL of PBS were transferred into 4- to 6-week-old B6/nude mice via tail vein injections, as previously reported (11). Four weeks after injection, monoclonal antibodies from hybridoma supernatants derived from splenocytes of recipient B6/nude mice were subjected to IFA to measure the production of ANA and anti-dsDNA autoantibodies using HEp-20-10/primate liver tissue and *Crithidia luciliae* substrate (1:10 dilution; Euroimmun, Lübeck, Germany), respectively. Fluorescence intensity was scored as follows: 0, no specific staining; 1, weakly positive staining; 2, intermediate positive staining; 3 strongly positive staining, as described previously (9).

2.6 Histologic analysis of hepatic inflammation

Liver tissues were obtained from scurfy mice and WT littermates at day 21 of life during routine necropsies and fixed in 4% neutral buffered formalin at 4°C overnight and then embedded in paraffin. 5 µm thick sections were stained with hematoxylin and eosin (H&E), according to standard protocols. For histological evaluation, periportal/periseptal interface hepatitis and portal inflammation were scored using the following criteria, originating and adapted from the modified Histological Activity Index (HAI) grading system by Ishak et al. (22): For periportal/periseptal interface hepatitis; grade 0 (absent), grade 1 (mild; focal, few portal areas), grade 2 (mild/moderate; focal, most portal areas), grade 3 (moderate; continuous around <50% of tracts or septa), grade 4 (severe; continuous around >50% of tracts or septa). For portal inflammation; grade 0 (none), grade 1 (mild; some or all portal areas), grade 2 (moderate; some or all portal areas), grade 3 (moderate/marked; all portal areas), grade 4 (marked; all portal

areas). The final inflammation grade (0-4) was dictated by the higher score of both categories.

2.7 Collagen quantification

75 mg of liver tissue were taken from the left lobe between 15 and 25 days of life. Samples were hydrolyzed in screw-capped polypropylene tubes (Greiner Bio-One, Frickenhausen, Germany) in 6 N HCl (1.25 mL per liver) at 110°C overnight, followed by centrifugation to remove solids and the supernatant collected. Triplicates of 5 µl of the supernatant were placed in a 96 well-plate and mixed with 50 µl of 0.1 M citrate buffer, pH 6.0, and 100 µl of 150 mg chloramine T dissolved in citrate buffer (0.1 M, pH 6.0) for a 30-min incubation at RT. Next, 100 µl of Ehrlich´s (1.25 g of dimethyl-benzaldehyde dissolved in distilled water) was added to the reaction mixture and incubated at 65°C for 30 min. Absorbance was measured at 550 nm in an Infinite M200Pro spectrophotometer (Tecan, Crailsheim, Germany). A standard curve using L-hydroxyproline standard (Merck, Darmstadt, Germany) was prepared to determine hydroxyproline (HYP) concentration (23).

2.8 Real-time quantitative PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) and 1 µg of total RNA was reverse transcribed into cDNA with the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's recommendations. Quantitative real-time PCR (qRT-PCR) was conducted using validated Taqman gene expression sets for mouse procollagen α1(I) (COL1A1) (Mm00801666_g1), α-smooth muscle actin (α-SMA, ACTA2; Mm00725412_s1), tissue inhibitor of metalloproteinases 1 (TIMP-1; Mm01341361_m1), transforming growth factor beta 1 (TGFβ1; Mm01178820_m1), tumor necrosis factor α (TNFα; Mm00443258_m1), interferon-γ (IFNγ; Mm01168134_m1), matrix metalloproteinase 2 (MMP-2; Mm00439498_m1), matrix metalloproteinase 9 (MMP-9; Mm00442991_m1), and matrix metalloproteinase 13 (MMP-13; Mm00439491_m1) (Life Technologies, Carlsbad, USA) on a Step One Plus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Beta-2 microglobulin (*B2m*) served as an endogenous control for internal normalization (24). Data were analyzed using the ΔΔ-Ct method, as described (25). The fold change was calculated as 2-ΔΔCt.

2.9 Isolation of hepatic non-parenchymal cells

Liver tissues were obtained from scurfy mice and WT controls between 15 and 25 days of life. Livers were minced and cells were treated with collagenase buffer (0.4% collagenase type IV, Sigma-Aldrich, St. Louis, MO, USA), 154 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 20.1 mM HEPES, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.6 nM DNase I (Applichem, Darmstadt, Germany) (pH 7.4) and dispersed with a gentle MACS dissociator (Miltenyi Biotec, Bergisch-Gladbach, Germany). Homogenates were incubated at 37°C for 30 min, passed through a 100 µm cell strainer (BD Bioscience, San Jose, CA, USA) and centrifuged at 21 ×g for 4 min in ice cold PEB buffer (PBS, 2 mM EDTA, 0.5% BSA). Supernatants were centrifuged at 300 ×g for 10 min and cell pellets were resuspended in PEB buffer. Red blood cells were lysed by adding 10 volumes of 150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA·2Na. Non-lysed immune cells were washed twice and suspended in PEB buffer, as previously described (26).

2.10 Flow cytometry

Non-parenchymal liver cells were blocked with the 2.4G2 anti-Fc receptor antibody (BD Bioscience, San Jose, CA, USA) and stained with antibodies recognizing CD11b, CD11c, CD45, F4/80, Gr-1, Ly-6C (BD Bioscience, San Jose, CA, USA; Biolegend, San Diego, CA, USA; eBioscience, San Diego, CA, USA). For intracellular staining, cells were fixed in Fix/Perm buffer (BD Bioscience, San Jose, CA, USA), washed in PBS containing 2% goat serum and incubated in Perm/Wash buffer (BD Bioscience, San Jose, CA, USA) with an anti-CD206 antibody serving as M2 macrophage marker (eBioscience, San Diego, CA, USA). Cell acquisition was conducted on a fluorescence-activated cell sorting (FACS) Canto II (BD Bioscience, San Jose, CA, USA) and analyzed with the FLOWJO software (TreeStar, Ashland, OR, USA), as previously reported (26). The gating strategy was as follows: Neutrophil subsets were enriched from viable $CD45^+$ and $Ly6G^+$ immune cells. Total hepatic macrophages were obtained by subsequent enrichment of viable subset of $CD45^+$ and $Ly6G^-$ immune cells and were separated into monocytic and resident macrophage subsets by gating separately for CD11b and F4/80. Monocytic macrophages from $CD11b^{hi}$ $F4/80^{int}$ subset were again subdivided regarding their expression of Ly-6C. Resident macrophages from $CD11b^{int}$ $F4/80^{hi}$ subset were further subdivided by the expression of CD11c and CD206 (Supplementary Figure 1A).

2.11 Statistical analysis

Results are expressed as mean ± SD. Differences were analyzed by Mann-Whitney U test, if not indicated otherwise. Significance was determined using Prism (GraphPad Software, La Jolla, USA) and $p < 0.05$ were considered significant. (∗) represents $p < 0.05$, (∗∗) represents $p < 0.01$, (∗∗∗) represents $p < 0.001$ and (∗∗∗∗) represents $p < 0.0001$. Rank-based non-parametric trend analysis of independent samples was performed with Jonckheere-Terpstra test using R Statistical Software (v4.3.1, URL: https://r-project.org) via clinfun package, and the corresponding figure was created using the ggbeeswarm and ggpubr packages.

3 Results

3.1 Scurfy mice produce ANA against AILD-specific and related antigenic targets

ANA are serological hallmarks of a wide range of autoimmune diseases including those that manifest AILD, most importantly AIH (27). Therefore, we initially screened sera of scurfy mice for the existence of ANA by IFA. All scurfy mice (n=20) produced ANA with end-point titers ranging from 1:100 to 1:1000, whereas ANA were found in only 15% of healthy littermates (3/20) (Figures 1A, B). In scurfy mice, the most frequently (in 80%) observed IFA staining pattern on HEp-20-10 cells was the nuclear coarse speckled pattern designated AC-5 according to ICAP nomenclature (17), followed by AC-18 (cytoplasmic discrete dots/GW body-like, in 60%) as well as AC-15 and AC-16 (cytoplasmic fibrillar linear and cytoplasmic fibrillar filamentous, in 30%, respectively) (Figure 1C). In the majority of scurfy sera, multiple IFA patterns occurred simultaneously (in 85%, data not shown), which suggests a polyclonal humoral response. In order to further distinguish specific patterns, hybridoma cell lines of activated plasma cells from scurfy mice (n=2) were generated and tested for ANA positivity until a singular IFA staining pattern was reached by repeated subcloning. Consistently, AC-15 and AC-18 patterns were detected (Figure 1D), which are found to be associated with AIH-1 and PBC, respectively (28, 29).

As each AILD entity is characterized by a distinct serological profile, we subsequently determined the target antigens of AILD-specific autoantibodies. Interestingly, autoantibodies against all these targets were observed at significantly higher levels in scurfy sera compared with WT sera (Figure 2; Supplementary Table 1). AMA were the most prevalent autoantibody specificity in scurfy mice (in 82.6%), followed by anti-VCP (in 65.22%) and anti-gp210 autoantibodies (in 52.2%). Since AC-18 was the second most common pattern, we additionally performed a serological analysis for associated autoantibodies against EEA1, Ge-1, GW182, and Ago2 ("cytoplasmic dot profile"). Similarly, a significantly higher production was noted for all four autoantibodies in scurfy

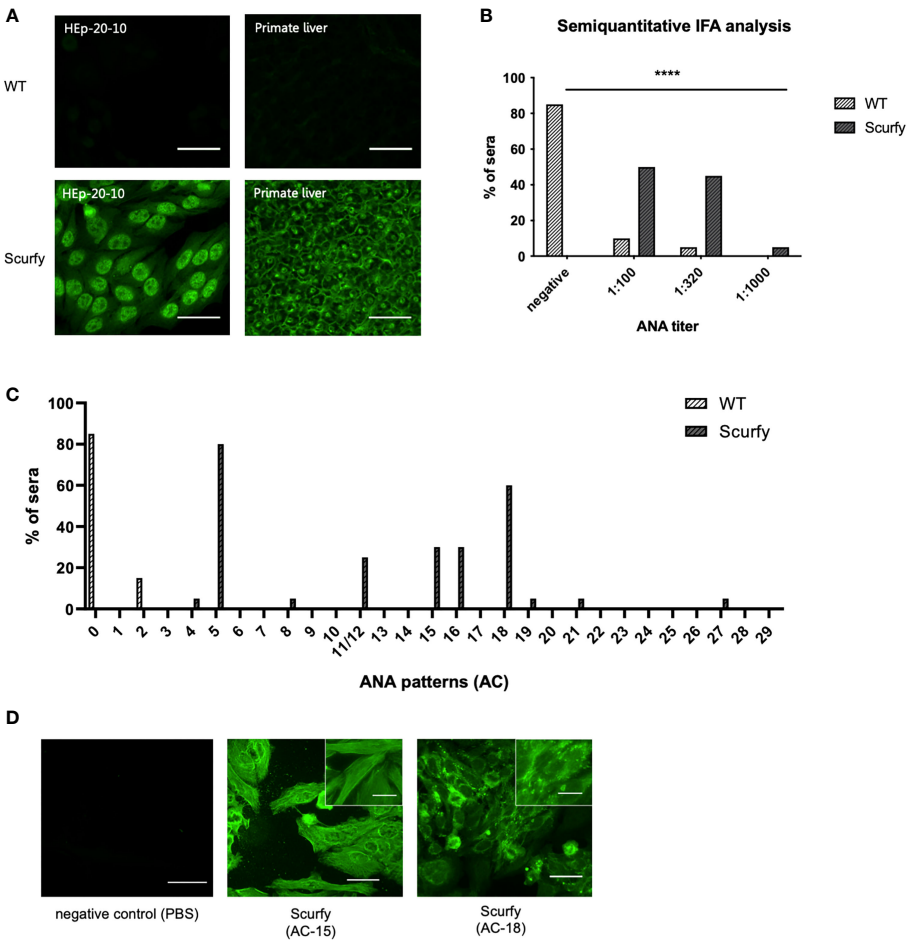

FIGURE 1
Scurfy mice produce ANA in the absence of Treg. **(A)** Representative images by IFA on HEp-20-10 cells and primate liver tissue with WT (upper panel) and scurfy (lower panel) serum. Scale bars, 50 µm. **(B)** Semiquantitative IFA analysis with ANA titers in sera of WT and scurfy mice ****p<0.0001 (Mann-Whitney U test). **(C)** Analysis of ANA patterns in WT and scurfy sera by using designated codes from AC-0 (negative) to AC-29, according to ICAP nomenclature (scurfy n=20, WT n=20). **(D)** Representative images by IFA on HEp-20-10 cells with hybridoma supernatants directly obtained scurfy mice. Cytoplasmic fibrillar linear pattern (AC-15, middle panel), cytoplasmic discrete dots/GW-body like pattern (AC-18, right panel), and negative control (left panel) are shown. Scale bars, 50 µm (larger images) and 20 µm (close-up views).

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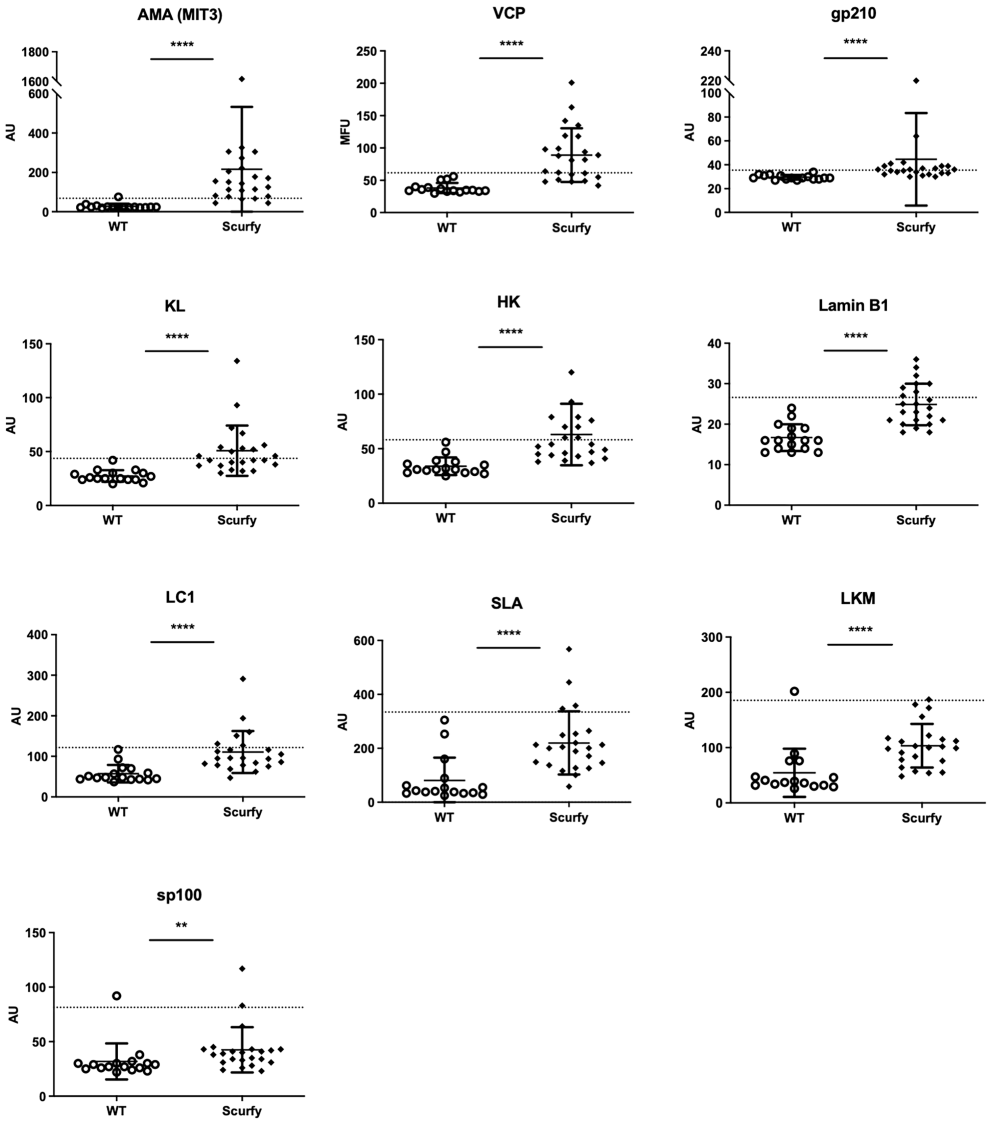

FIGURE 2
Scurfy mice exhibit AILD-associated autoantibodies. Detailed analysis of autoantibodies against antigenic targets associated with or related to AILD by using LIA, ELISA, and ALBIA. For ALBIA, titers are expressed in median fluorescent units (MFU). For LIA and ELISA, values are expressed in absorbance units (AU). Dashed lines represent cutoff values established at three SD over the mean of WT controls (scurfy n=23, WT n=16). **p<0.01, ****p<0.0001 (Mann-Whitney U test). Data are expressed as mean ± SD.

mice, especially for anti-EEA1 autoantibodies (Figure 3; Supplementary Table 2).

3.2 Autoreactive CD4$^+$ T cells from scurfy mice induce ANA production with AILD-associated fluorescence pattern in B6/nude mice

To assess if autoreactive CD4$^+$ T cells are sufficient to induce production of AILD-related autoantibodies through T-cell dependent B-cell activation, CD4$^+$ T cells of scurfy mice were adoptively transferred into T cell-deficient *nu/nu* mice. Remarkably, hybridoma supernatants obtained from splenocytes of all recipient *nu/nu* mice (n=6) revealed ANA positivity, whilst only one mouse showed anti-dsDNA autoantibodies (Figures 4B–D). Of note, one of the recipient mice yielded a nuclear envelope staining pattern (AC-11/12) (Figure 4A), which is strongly associated with AILD, particularly PBC (30).

3.3 Scurfy mice spontaneously develop portal inflammation with interface hepatitis and cholangitis

The histopathology of a liver biopsy is a major diagnostic criterion for most AILD as defined by disease-specific alterations of hepatocytes and/or biliary epithelium. Accordingly, we analyzed histomorphologic features of liver samples obtained from scurfy mice and WT controls.

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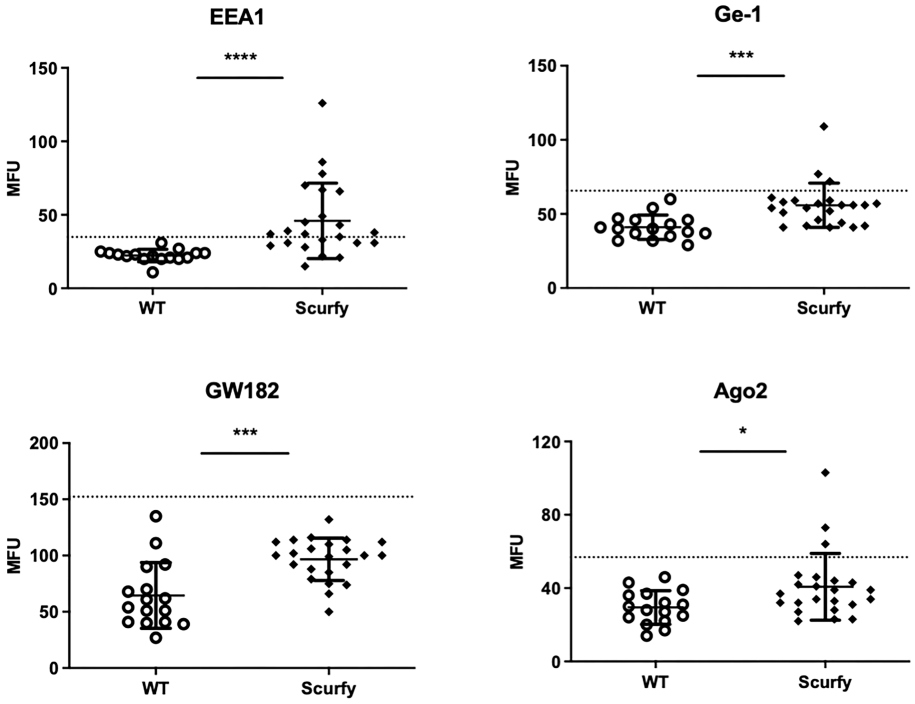


FIGURE 3
Production of autoantibodies against GW bodies in scurfy mice. Assessment of "cytoplasmic dot profile" by ALBIA in sera of WT and scurfy mice. Values are expressed in median fluorescent units (MFU). Dashed lines represent cutoff values established at three SD over the mean of WT controls (scurfy n=23, WT n=16). *p<0.05, ***p<0.001, ****p<0.0001 (Mann-Whitney U test). Data are expressed as mean ± SD.

Notably, all scurfy mice revealed significant lymphoplasmacytic infiltration of enlarged portal tracts and degeneration and proliferation of interlobular bile ducts (Figures 5B, C), a feature typically seen in PBC. Moreover, AIH-like interface hepatitis and necroinflammatory activity in hepatic parenchyma were present (Figures 5B, C). Multifocal interspersed erythroblastic islands were also detected in hepatic parenchyma, indicating extramedullary hematopoiesis (Figure 5C), as previously reported (31). In line with previous findings (31), no granulomas were found. Overall, one scurfy mouse (8.33%) displayed a severe hepatic inflammation (grade 4), whereas grade 3 and grade 2 inflammation was observed in seven (58.33%) and four (33.33%) scurfy mice, respectively (Figure 5D). In contrast, no relevant inflammatory or degenerative anomalies were observed in the liver of WT mice (Figure 5A). A rank-based trend analysis revealed no significant correlation between serum levels of AILD-associated autoantibodies and histopathologic liver disease score in scurfy mice (Supplementary Figure 2), which might be attributed to the small sample size (n=12).

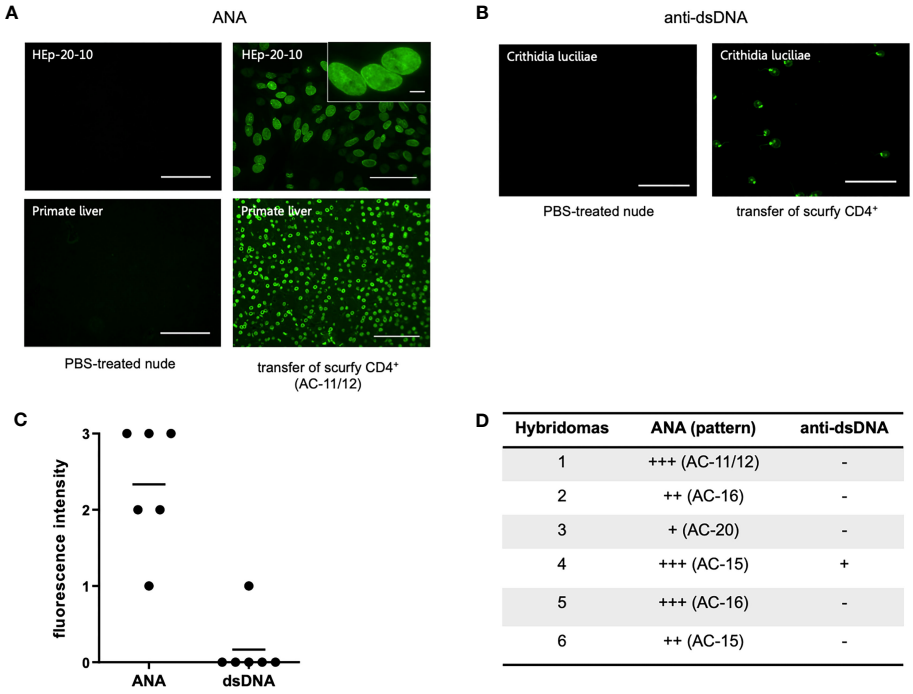
3.4 Profibrogenic transcripts are upregulated in the liver of scurfy mice

Liver fibrosis is characterized by the formation of a fibrous scar due to progressive accumulation of extracellular matrix (ECM) proteins, predominantly collagens (32). The gold standard to assess collagen deposition in fibrotic liver is through quantification of hydroxyproline (HYP), an amino acid unique to collagen (33, 34). In this regard, we measured HYP levels in scurfy und WT mice and found no significant changes in the total hepatic collagen content (Figure 6B), although the liver weights of scurfy mice were significantly increased (Figure 6A).

Subsequently, transcript levels of genes associated with hepatic fibrosis were assessed via qRT-PCR, which revealed a significant upregulation of fibrosis-related transcripts encoding proteins such as procollagen α1(I), TIMP-1, α-SMA (ACTA2), MMP-2, and MMP-9 in the livers of scurfy mice. Furthermore, a significant expression of TNFα was observed in the scurfy liver, contributing to the inflammatory environment in the hepatic tissue (Figure 6C).

3.5 Scurfy liver exhibits a proinflammatory phenotype with increased numbers of monocytic macrophages (Ly-6C^{hi})

Monocyte-macrophages are not only key regulators in the maintenance, progression, and reversal of liver fibrosis, but also contribute to the pathogenesis of AILD (35). Therefore, we next focused on the cellular composition of the inflammatory infiltrates in liver of scurfy mice. Flow cytometry analysis revealed a proinflammatory phenotype in the absence of collagen accumulation. The number of monocytes and neutrophils was markedly elevated in scurfy liver tissue, whereas macrophages were detected at lower levels compared to WT controls



Hybridomas	ANA (pattern)	anti-dsDNA
1	+++ (AC-11/12)	-
2	++ (AC-16)	-
3	+ (AC-20)	-
4	+++ (AC-15)	+
5	+++ (AC-16)	-
6	++ (AC-15)	-

FIGURE 4
Transfer of scurfy CD4$^+$ T cells induces production of ANA and anti-dsDNA autoantibodies in recipient B6/nude mice. **(A)** Representative images by IFA on HEp-20-10 cells and primate liver showing the nuclear envelope pattern (AC-11/12, right panel) in hydridomas from B6/nude mice after transfer of scurfy CD4$^+$ T cells, while sera of PBC-treated nude mice were negative for ANA (left panel). Scale bars, 100 µm (left panel and right lower panel), 50 µm (right upper panel), 10 µm (close-up view). **(B)** Representative IFA staining on *Crithidia luciliae* substrate of monoclonal antibody from hybridomas derived from B6/nude mice transferred with scurfy CD4$^+$ T cells, demonstrating anti-dsDNA positivity (right panel). No anti-dsDNA production was induced in nude mice by PBS injection (left panel). Scale bars, 50 µm. **(C)** Screening of monoclonal autoantibodies from hybridoma supernatants derived from splenocytes of recipient B6/nude mice for ANA and anti-dsDNA (n= 6). **(D)** Tabular summary of the IFA screening of hybridoma supernatants from recipient B6/mice after transfer of scurfy CD4$^+$ T cells.

(Figure 7A). Importantly, we identified increased inflammatory monocytic macrophages (Ly-6C^{hi}) and reduced restorative monocyte populations (Ly-6C^{lo}) in livers of scurfy mice (Figure 7B). Regarding liver-resident macrophages, increased proinflammatory M1 (CD11c$^+$) and reduced M2 (CD206$^+$) macrophages were detected in scurfy mice (Figure 7C; Supplementary Figure 1).

4 Discussion

AILD are immune-mediated chronic inflammatory disorders characterized by an abrogation of peripheral tolerance against hepatocytes and biliary epithelium (36). Screening for disease-related autoantibodies is essential to facilitate the diagnosis of AIH and PBC, whilst being of relatively minor importance in PSC. Although AIH is widely considered a T-cell mediated disease and the pathogenic role of autoantibodies remains ill-defined, several autoantibodies have been linked to different clinical phenotypes. As such, type 1 AIH (AIH-1, classic type) is associated with ANA and anti-SMA, whereas the rarer, yet more aggressive forms AIH-2 and AIH-3 are defined by the existence of anti-LKM/anti-LC1 and anti-SLA autoantibodies, respectively (12, 37–39). Considering the severe course of disease in scurfy mice, it is important to note that a significantly increased production of anti-LKM, anti-LC1, and anti-SLA autoantibodies was detected in sera of scurfy mice, along with ANA (Figures 1, 2).

AMA, a serological hallmark of PBC, were by far the most prevalent AILD-associated autoantibodies in scurfy mice (in 82.6%, Figure 2), supporting previous findings (31). This is in accord with the predominant cholangitis in scurfy liver (Figure 5), while interface hepatitis and necroinflammatory activity in hepatic parenchyma, as seen in AIH, were observed to a lesser extent (Figure 5). Scurfy mice also revealed significantly elevated levels of anti-gp210 autoantibodies, which are reported to be associated with worse prognosis and higher risk at hepatic failure in PBC (40, 41). More recent evidence suggests that patients with anti-gp210 antibodies were more likely to develop interface hepatitis and lobular inflammation, akin to that in scurfy mice and PBC-AIH overlap syndrome (20, 31, 40). This concurs well with a study demonstrating that the prevalence of anti-gp210 autoantibodies was significantly higher in PBC-AIH overlap syndrome than in PBC and AIH (42). Furthermore, two recently identified biomarkers for PBC, anti-KL and anti-HK autoantibodies, were detected in almost half of scurfy sera (Figure 2). To date, little is known about clinicopathological correlations of these novel autoantibodies, although there is increasing evidence that anti-HK1 autoantibodies may be affiliated with poorer prognosis in PBC (43). Since cytoplasmic dot staining was frequently observed in scurfy sera by IFA, we opted for determination of autoantibodies

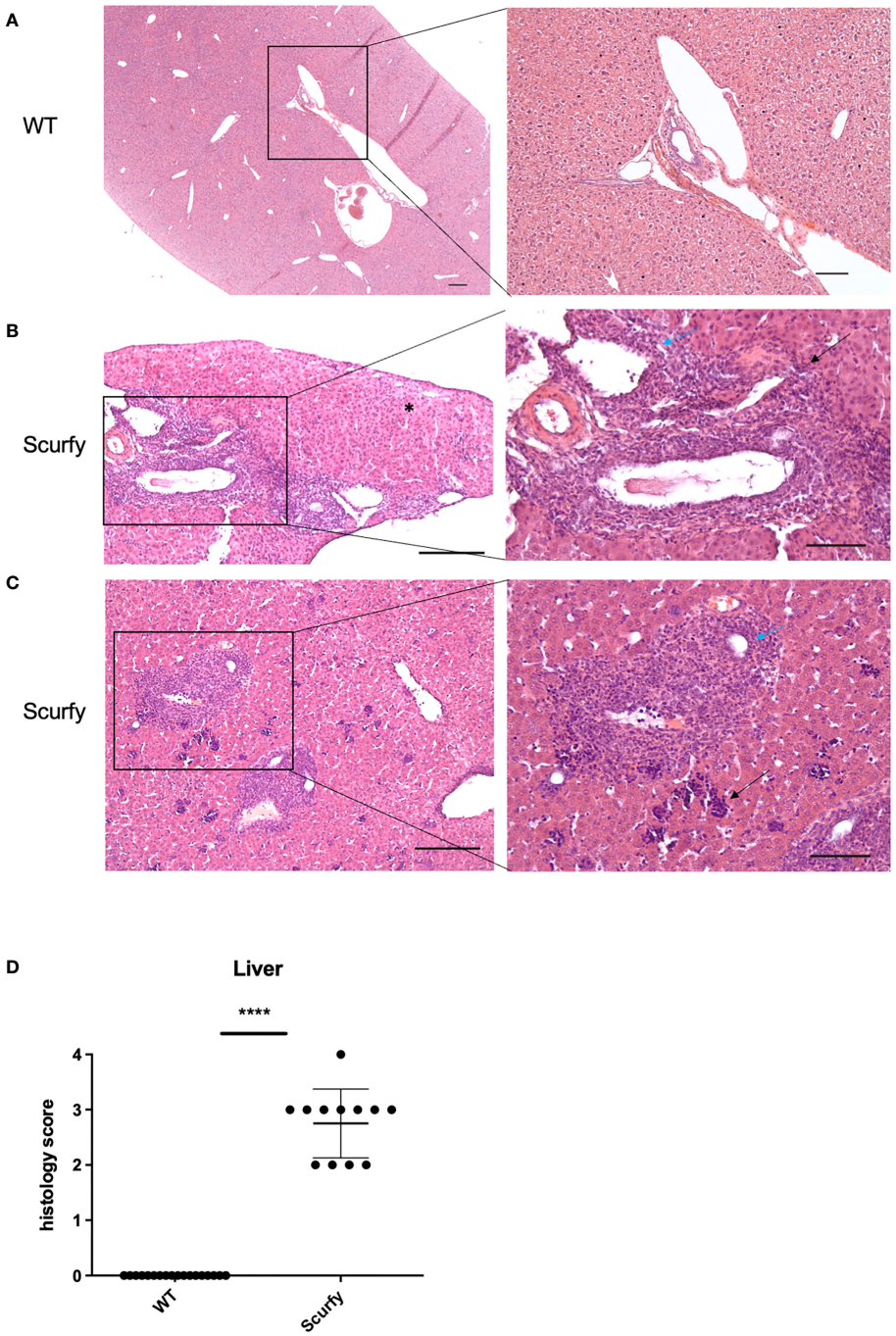


FIGURE 5
Scurfy mice spontaneously develop portal inflammation and interface hepatitis. **(A)** H&E staining of normal liver tissue of WT mice. **(B)** Severe lymphoplasmacytic infiltrates in enlarged portal tracts are shown. Necroinflammatory activity in hepatic parenchyma (asterix, left panel), interface hepatitis (black arrow, right panel), and lymphocytic infiltration of biliary epithelium of an irregular bile duct (blue arrow, right panel) were presented. **(C)** Marked inflammation of the portal tract including degeneration of the interlobular bile duct (blue arrow, right panel). Multifocal interspersed erythroblastic islands in hepatic parenchyma (black arrow, right panel). Scale bars, 200 µm (left panel) and 100 µm (right panel). **(D)** Grading of inflammation in scurfy and WT liver (scurfy n=12, WT n=18), ****p<0.0001 (Mann-Whitney U test). Data are expressed as mean ± SD.

against distinct cytoplasmic domains known as GW (G (glycine) W (tryptophan)-containing) bodies. These comprise unique cytoplasmic foci which exert critical functions in mRNA processing in the microRNA pathways and have been recently proposed as complementary biomarkers for PBC found in 5-10% of affected patients (19, 29, 44–46). In our study, scurfy mice yielded a significant production of autoantibodies against several GW bodies, i.e., GW182/TNRC6, Ago2, and Ge-1 (Figure 3). Interestingly, these structures do not possess a surrounding membrane, which may make them readily targetable by autoantibodies (19), as opposed to most of the other intracellular autoantigens in AILD which might only be released upon

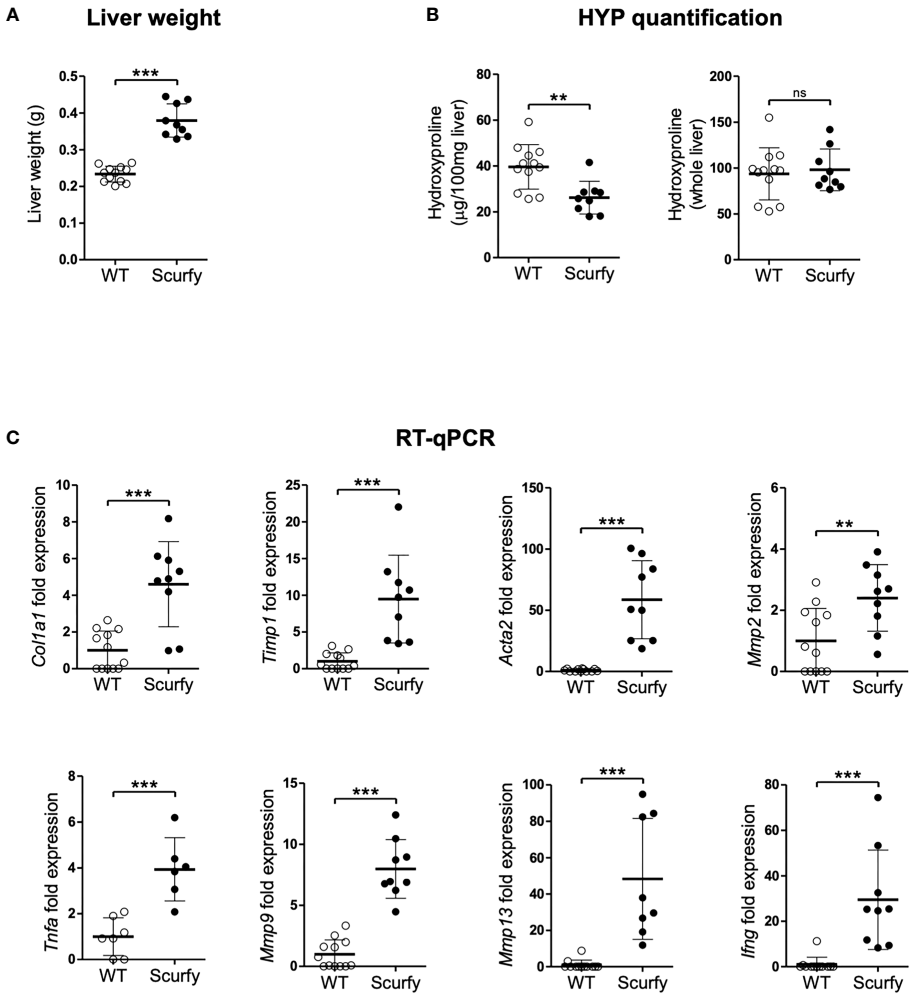


FIGURE 6
Upregulated profibrogenic transcripts in the absence of collagen accumulation in scurfy mice. **(A)** Assessment of liver weight in scurfy and WT mice. **(B)** HYP content per 100 mg liver (relative HYP, left panel) and per whole liver (total hepatic HYP, right panel) in scurfy and WT mice. **(C)** Levels of fibrosis-related transcripts in liver tissue of scurfy and WT mice (scurfy n=9, WT n=12). ns: not significant, **p<0.01, ***p<0.001 (unpaired Student's t-test). Data are expressed as mean ± SD. Results are representative of ≥2 independent experiments.

hepatocyte damage. By comparison, EEA-1 is part of the endosome/phagosome pathways with an IFA staining pattern that can resemble that of GW bodies, but comparatively little is known about its clinical associations (47).

Overlap syndromes are estimated to account for up to 20% of patients with PBC, the majority displaying features of both PBC and AIH (48, 49). Previous reports indicate that PBC-AIH overlap syndrome differs from isolated forms concerning disease course, prognosis, and therapeutic responses (50). Serologically, PBC-AIH overlap syndrome is generally defined by the presence of key autoantibodies of PBC and/or AIH, i.e. AMA and anti-SMA autoantibodies, and usually higher levels of transaminases and relative therapy resistance according to the Paris criteria (51). Recent studies, however, suggest that the serological profile of PBC-AIH overlap syndrome seems to be much more complex. Not only were other AILD-associated autoantibodies detected in this entity, including those against GW bodies, LKM, SLA, HK, gp210, KL, and sp100, but also distinct disease-specific serological patterns were reported (20). In this regard, anti-dsDNA autoantibodies have generated considerable interest since the concomitant existence of AMA and anti-dsDNA has been proposed to be highly specific for AIH-PBC overlap syndrome (52). This corroborates with our transfer experiments showing that autoreactive $CD4^+$ T cells from scurfy mice were sufficient to activate B cells of recipient B6/nude mice to produce anti-dsDNA autoantibodies along with the AILD-associated AC-11/12 fluorescence pattern (Figure 4). Anti-dsDNA autoantibodies are typically regarded as serological biomarkers of systemic lupus erythematosus (SLE) (53). While it is assumed that autoantibodies in PBC-AIH overlap syndrome might target unique dsDNA epitopes, there is also evidence indicating an association between PBC and SLE as these entities overlap in some patients (20, 54). A higher incidence of CTD in AIH was also reported (55). We have previously shown that scurfy mice exhibit features of CTD, including SLE, scleroderma, and mixed connective tissue disease (9, 10, 56). Further studies are required to

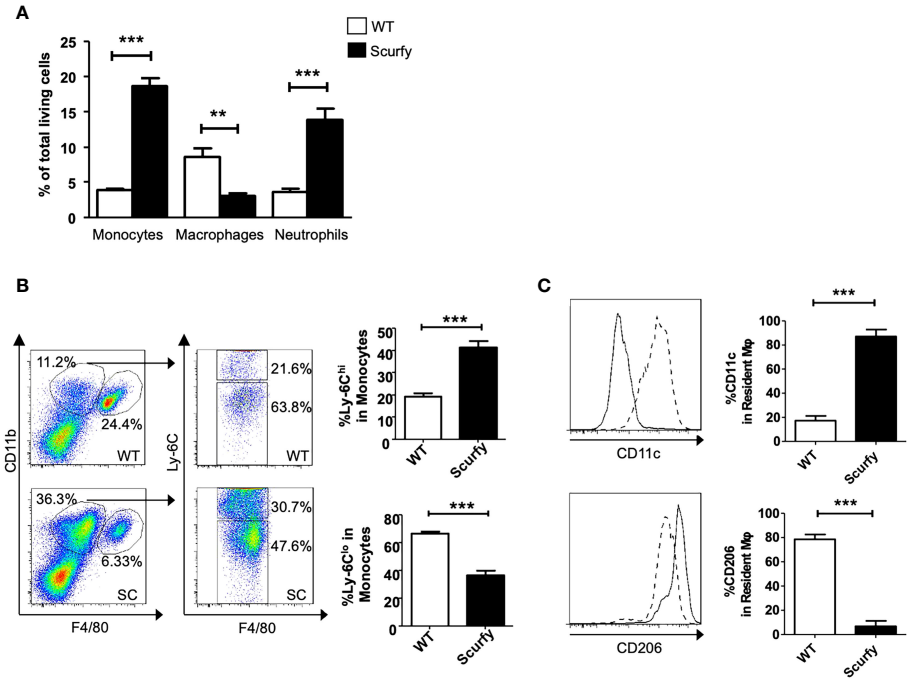


FIGURE 7
Increased pro-inflammatory and reduced restorative monocytes and macrophages in scurfy mice. **(A)** Bar graph indicating percentage of monocytes, macrophages and neutrophils in total living cells of WT and scurfy mice. **(B)** Representative FACS plots showing monocyte ($CD11b^{hi}$ $F4/80^{lo}$) and macrophage ($CD11b^{int}$ $F4/80^{hi}$) population (left panel). Monocytes were further analyzed by the differential expression of Ly-6C (right panel). Bar diagrams demonstrate the percentage of Ly-$6C^{hi}$ and Ly-$6C^{lo}$ population from monocytes. **(C)** Representative histograms showing the expression of CD11c and CD206 in macrophages. Solid line indicates WT mice; dot line indicates scurfy mice. Bar diagrams show the percentage of CD11c and CD206 in resident macrophages (*p<0.05, **p<0.01, ***p<0.001 unpaired Student's t-test; scurfy n=6, WT n=6).

investigate the role of functional Treg deficiency as a common pathway in patients with concurring AILD and CTD. Given that anti-dsDNA autoantibodies were only detected in one out of six recipient B6/nude mice (16.67%) in the current study, the results should be interpreted with caution and need to be validated by future studies. Nevertheless, the proportion of B6/nude mice with anti-dsDNA production seems to reflect the proportion of autoreactive $CD4^+$ T cells transferred from scurfy mice which are able to induce autoantibody production in the recipient B cell population, as anti-dsDNA autoantibodies were previously found to be prevalent in 15% of scurfy mice (10).

In this study, a wide range of AILD-related autoantibodies in sera of scurfy mice was detected (Figures 2, 3). This finding can be explained by a polyclonal humoral response due to the uncontrolled expansion of $CD4^+$ T cells in the absence of Treg, leading to production of more than a single autoantibody. On the other hand, the relationship between PBC and AIH in an overlapping setting is believed to be multilayered, ranging from sequential presentation of both entities, their simultaneous existence to being part of a disease continuum (57). As such, detection of multiple autoantibodies both in general and in the same patient has been reported in PBC-AIH overlap syndrome (20, 52). An important question to resolve for future research is why some autoantibodies (e.g., AMA, anti-VCP, anti-EEA1) were produced in the majority of scurfy mice, whereas others (e.g., anti-GW182, anti-LKM) were relatively rare.

As crucial effector cells of innate immunity, macrophages play an important role in the hepatic microenvironment via polarization to different phenotypes (classically activated M1-type and a spectrum of alternatively activated M2-type macrophages) under pathological conditions such as liver fibrosis, viral hepatitis, and hepatocellular carcinoma (35). The past five years have witnessed a renewed importance of macrophages in AILD as well. M1- and M2-type peribiliary macrophages were shown to be increased in human and murine PSC, while M1-type macrophages have been associated with enhanced Notch signaling and self-renewing phenotypes of hepatic progenitor cells (58, 59). In a concanavalin A (ConA)-induced AIH mouse model, splenectomy and IL-34 were found to drive M2 polarization which suppressed hepatic fibrosis and inflammation (60, 61). Furthermore, Li et al. demonstrated that cholangiocyte-derived exosomal long noncoding RNA H19 promoted M1 polarization and hepatic inflammation in PBC and PSC (62). These results indicate that M1 polarization seems to exacerbate AILD, whereas an M2-type polarization promotes inflammation resolution [reviewed in (35)]. This substantiates our findings in scurfy liver revealing a strong macrophage differentiation towards the M1 phenotype and a decreased M2-type polarization in a TNFα-dominated proinflammatory microenvironment (Figures 6, 7) Surprisingly, this contrasts with our previous research which showed M2-polarized macrophages and a significant Th2 deviation in the skin of scurfy mice, as partly found in scleroderma (56). An explanation for these rather

divergent responses may be that scurfy mice possibly recapitulate different stages of PBC-AIH overlap syndrome and CTD. This finding can also be attributed to potential differences in the cutaneous and hepatic inflammatory milieu. Further studies, including single-cell profiling, are needed to determine the heterogeneity and functional plasticity of macrophages in PBC-AIH overlap syndrome and organ-specific inflammation patterns in scurfy mice. Although a significant upregulation of profibrogenic or ECM remodeling transcripts was observed in scurfy liver, no collagen accumulation was detected (Figure 6). The finding that total hepatic collagen accumulation, as quantified by liver HYP, was not increased at sacrifice in scurfy mice, despite highly elevated fibrosis-related transcript levels, can be explained by their short lifespan, which necessitated their sacrifice at 15 to 25 days of age. Thus, only chronic inflammation of "wounds that do not heal" will lead from a Th1 T cell and M1-type fibrolytic immune cell response to a less inflammatory Th2 T cell and M2-type macrophage dominated response that drives tissue fibrosis (63–66). This would likely have been the case in scurfy mice if they would live longer than four weeks, as would be expected to occur in immune deficient patients (63–66). This is also in line with previous findings that fibrosis phenotypes, e.g., collagen accumulation, are significantly detected, chemically or macroscopically, after four weeks of age in most mouse models for liver fibrosis. In this context, at least four weeks are required for a statistically significant increase of collagen deposition in the carbon tetrachloride (CCL_4)- and thioacetamide (TAA)-induced fibrosis model, which is the most representative mouse model for panlobular fibrosis (23, 67). An even slower fibrosis progression was noted for the biliary fibrotic *Mdr2* knockout mice which displays a significant hepatic collagen accumulation after ten weeks of age (23, 68). This observation was also reported in other age-dependent fibrosis development studies using genetically modified mouse strains in which the upward trend of hepatic HYP levels was clearer at four to eight weeks after birth (68). We therefore hypothesize that the early-onset lethal scurfy phenotype might represent a premature model for the full development of fibrosis and/or that their specific Foxp3 defect may favor both active fibrogenesis and fibrolysis, resulting in no significant net fibrosis. This can be supported by previous findings that MMP-9 and MMP-13, for instance, can be both fibrogenic via tissue remodeling and subsequent repair, but also be fibrolytic in other (resolution) settings (63, 69). Our findings further suggest a potential link between macrophages and $CD4^+$ T cell-mediated tissue destruction in scurfy mice. In the aforementioned study by Haeberle et al., spontaneous Th2 cytokine secretion of skin infiltrating $CD4^+$ T cells was associated with M2-polarized macrophages in the skin (56). Concordantly, scurfy mice were found to exhibit cartilage degradation and nonerosive arthritis in the paws, comprising $CD3^+$ T lymphocytes, B cells, but also neutrophiles and macrophages (9). As previously demonstrated, in liver tissue of scurfy mice, $CD4^+$ T cells were predominantly accumulated in periportal areas, while cytotoxic $CD8^+$ T cells were concentrated around the bile ducts (31). The same study also identified increased expression of hepatic genes encoding cytokines such as IL-12, which is mainly produced by antigen-presenting cells including dendritic cells, monocytes, and macrophages (31, 70).

Previous reports indicate that, compared to isolated PBC, PBC-AIH overlap syndrome leads to significantly higher rates of unfavorable outcomes including esophageal varices, gastrointestinal bleeding, ascites, need for liver transplant, as well as abatement of 5-year survival (50, 71). Thus, there is an unmet need for therapeutic options which specifically target key molecules in the pathogenesis of AILD. Notably, a growing body of literature demonstrates safety and efficacy of adoptive Treg therapy in a variety of immune-mediated diseases (72, 73). However, available data on the precise role of Treg in AILD have been sparse and in part contradictory. In AIH patients, recent reports have suggested that Treg are fully functional and are not reduced in frequency (74, 75), despite initial studies indicating otherwise [reviewed in (73)]. Some researchers even found increased intrahepatic and peripheral Treg frequency in AIH, which was more prominent in pediatric patients than adults, implicating that intrahepatic Treg might be functionally defective or insufficient for disease control (75–77). Of note, a 4-year-old patient with IPEX syndrome was reported to develop AIH-2 with anti-LKM1 autoantibodies, which points to involvement of Treg in pathogenesis of AIH (78). In contrast, PBC has been clearly shown to be associated with functionally and numerically impaired Treg both in peripheral blood and liver of patients and mouse models (79–81). Further studies have highlighted the significance of Treg in PBC by demonstrating that deficiency in the alpha subunit of the IL-2 receptor (IL2RA; CD25) led to PBC-like liver disease in human und murine setting (82, 83). Importantly, healthy sisters and daughters of PBC patients were found to possess a significantly reduced Treg frequency, indicating a genetic susceptibility to Treg deficiency in PBC (84). In this context, our results might serve as an incentive for future research to determine the functionality and frequency of Treg in PBC-AIH overlap syndrome.

In summary, we confirm and extend previous findings on PBC-like liver disease in scurfy mice and provide further evidence of concomitant aspects typical of AIH. Our results support the hypothesis that Treg deficiency and the consequent breach of humoral tolerance in scurfy mice is key to the spontaneous development of clinical, serological, and immunopathological features of AILD with overlapping characteristics of PBC and AIH. The study sheds a new light on the role of Treg in the pathogenesis of immune-mediated liver disease.

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 approved by Regierungspräsidium Karlsruhe, Germany. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KY: Visualization, Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. SH: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – review & editing, Investigation. YK: Visualization, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. MF: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing, Supervision. S-YW: Visualization, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. BG: Formal Analysis, Investigation, Methodology, Writing – review & editing. VR: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Writing – review & editing. KS: Project administration, Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. DS: Formal Analysis, Project administration, Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. AE: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. EH: Supervision, Writing – review & editing, Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources.

Funding

The present work was supported from a German Research Foundation (DFG)-Transregio-Grant to EH and AE (TRR-156/C04-246807620), KS (TRR-156/A04-246807620, TRR-156/C05-246807620), and VR, KS, and DS (TRR-156/C05-246807620), and by the DFG to KS (SFB1009/B11-194468054, SFB1066/B06-213555243, SFB1450/C06-431460824). DS received project-related funding from the DFG Collaborative Research Center (CRC) grants SFB 1066 project B3 (213555243) and CRC 1292 project B10, and by EU Horizon 2020 project under grant nr. 777377 (LITMUS, Liver Investigation on Marker Utility in Steatohepatitis). We acknowledge financial support by the DFG within the funding programme Open Access Publishing, by the Baden-Württemberg Ministry of Science, Research and the Arts and by Ruprecht-Karls-Universität Heidelberg.

Acknowledgments

The authors are indebted to Britta Heckmann and Stefanie Martinache (Heidelberg, Germany), Claudia Braun and Kyoung-Sook Park (Mainz, Germany) as well as Meifeng Zhang (Calgary, Canada) for excellent technical assistance. We thank Dr. Andreas Recke (Lübeck, Germany) for his support on part of the statistical analysis.

Conflict of interest

MF is the medical director of Mitogen Diagnostics Corporation.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1253649/full#supplementary-material

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10.3389/fimmu.2023.1253649

Glossary

ACTA2 (α-SMA)	α-smooth muscle actin
ALBIA	addressable laser bead immunoassay
AC	anti-cellular
Ago2	argonaute protein
AMA-MIT3	antimitochondrial antibodies against PDC-E2, BCOADC-E2, and OGDC-E2
ANA	antinuclear antibodies
AIH	autoimmune hepatitis
AILD	autoimmune liver diseases
COL1A1	procollagen α1(I)
CTD	connective tissue disease
ECM	extracellular matrix
EEA1	early endosomal antigen 1
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FoxP3	Forkhead Box 3
gp210	glycoprotein-210
GW	G (glycine) W (tryptophan)-containing
GW-182	glycine-tryptophan protein of 182 kDa
HEp-20-10	human epithelial cells
HK	hexokinase
HYP	hydroxyproline
H&E	hematoxylin and eosin
ICAP	International Consensus on ANA Patterns
IFA	indirect immunofluorescence assay
IFNγ	interferon-γ
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
KL	Kelch-like
LC1	liver cytosol type 1
LIA	line immunoassay
LKM	liver kidney microsome
MMP	matrix metalloproteinases
PBC	primary biliary cholangitis
PSC	primary sclerosing cholangitis
qRT-PCR	quantitative real-time polymerase chain reaction
Treg	regulatory T cells
SLA	soluble liver antigen
SLE	systemic lupus erythematosus
sp100	soluble protein 100 kDa
TGFβ1	transforming growth factor beta 1
TIMP-1	tissue inhibitor of metalloproteinases 1
TNFα	tumor necrosis factor α
VCP	valosin-containing protein
WT	wildtype

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 19 October 2023
DOI 10.3389/fimmu.2023.1286251

OPEN ACCESS

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RECEIVED 31 August 2023
ACCEPTED 10 October 2023
PUBLISHED 19 October 2023

CITATION
Fukasawa T, Yamashita T, Enomoto A, Norimatsu Y, Toyama S, Yoshizaki-Ogawa A, Tateishi S, Kanda H, Miyagawa K, Sato S and Yoshizaki A (2023) The optimal use of tildrakizumab in the elderly via improvement of Treg function and its preventive effect of psoriatic arthritis. *Front. Immunol.* 14:1286251. doi: 10.3389/fimmu.2023.1286251

The optimal use of tildrakizumab in the elderly via improvement of Treg function and its preventive effect of psoriatic arthritis

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Introduction: As a form of precision medicine, this study aimed to investigate the specific patient population that would derive the greatest benefit from tildrakizumab, as well as the mechanism of action and efficacy of tildrakizumab in reducing the occurrence of psoriatic arthritis (PsA).

Methods: To achieve this, a multi-center, prospective cohort study was conducted, involving a population of 246 psoriasis patients who had not received any systemic therapy or topical finger therapy between January 2020 and April 2023. Two independent clinicians, who were blinded to the study, analyzed nailfold capillary (NFC) abnormalities, such as nailfold bleeding (NFB) and enlarged capillaries, as well as the incidence of new PsA. Additionally, the factors that determined the response of psoriasis after seven months of tildrakizumab treatment were examined. The study also examined the quantity and role of regulatory T cells (Tregs) and T helper 17 cells both pre- and post-treatment.

Results: The severity of psoriasis, as measured by the Psoriasis Area and Severity Index (PASI), was found to be more pronounced in the tildrakizumab group (n=20) in comparison to the topical group (n=226). At 7 months after tildrakizumab treatment, multivariate analysis showed that those 65 years and older had a significantly better response to treatment in those achieved PASI clear or PASI 2 or less (Likelihood ratio (LR) 16.15, p<0.0001; LR 6. 16, p=0.01). Tildrakizumab improved the number and function of Tregs, which had been reduced by aging. Tildrakizumab demonstrated significant efficacy in improving various pathological factors associated with PsA. These factors include the reduction of NFB, enlargement of capillaries, and inhibition of PsA progression. The hazard ratio for progression to PsA was found to be 0.06 (95% confidence interval: 0.0007-0.46, p=0.007), indicating a substantial reduction in the risk of developing PsA.

Discussion: Tildrakizumab's effectiveness in improving skin lesions can be attributed to its ability to enhance the number and function of Tregs, which are known to decline with age. Furthermore, the drug's positive impact on NFB activity and capillary enlargement, both of which are recognized as risk factors for PsA, further contribute to its inhibitory effect on PsA progression.

KEYWORDS

psoriasis, psoriatic arthritis, nailfold capillary, risk factors, tildrakizumab, regulatory T cells, elderly, prospective study

Introduction

Psoriasis is a prevalent chronic inflammatory skin condition that is characterized by various treatment options (1). The field of psoriasis treatment has seen significant advancements, with the availability of at least 11 biologics specifically designed for its management (2). These treatment options encompass both oral and topical medications. The therapeutic targets for psoriasis are diverse and include the utilization of anti-tumor necrosis factor-alpha (TNF-α) antibodies, anti-interleukin (IL)-12/23p40 antibodies, and anti-IL-17 antibodies. Among such a wide variety of treatment options, treatment is often difficult because each patient responds differently to the same therapeutic agent, and what works well in one patient is often ineffective in another. Therefore, it is required to bring the most promising or most effective treatment for an individual patient. Predicting treatment response in advance and providing the most promising or most effective treatment for an individual patient is called precision medicine (3), but at present, it is difficult to predict these treatment responses in advance. In particular, among anti-IL-23 antibodies, there are more than three biologics, each of which differs in whether it is a fully human or humanized antibody, and its affinity also differs in each drug (4). Tildrakizumab, a humanized monoclonal antibody that specifically targets IL-23p19, has shown potential therapeutic effects. However, the specific patient population that would benefit from tildrakizumab remains unknown. Previous clinical trials have not provided sufficient evidence to determine the efficacy of tildrakizumab in elderly patients, likely due to the limited number of participants in this age group (5). Therefore, the objective of this study was to investigate the effectiveness of tildrakizumab in patients as part of a precision medicine approach.

In recent years, especially in Western countries and Japan, society as a whole has been aging, and the question of how to provide medical care to the elderly has become a social issue (6). Regulatory T cells (Treg) are known to be decreased in psoriasis patients, and their suppressive capacity is also known to be reduced (7). As one of the mechanisms, IL-23 is known to act on Tregs and cause pathogenic conversion to IL-17 producing cells (8). Therefore, tildrakizumab, an anti-IL-23 antibody, is expected to reverse this process. It is also known that the number of Tregs is decreased and their suppressive capacity is also decreased in the elderly (9), but there are no reports yet on an elderly patient with psoriasis.

Psoriasis is often accompanied by a range of complications that have a substantial impact on the prognosis of patients (10). One significant comorbidity associated with psoriasis is psoriatic arthritis (PsA), which results in osteoclastic arthritis and significantly diminishes patients' quality of life (10). Dermatologists have a crucial role in diagnosing PsA as skin symptoms typically manifest years before the onset of PsA-related symptoms (10). Prompt treatment is particularly vital for PsA, as delays in treatment can lead to a decline in quality of life (11). Consequently, it is imperative to impede the progression to PsA. However, there are no reports on treatment effect of tildrakizumab for risk factors of progression to PsA, which includes NFB or enlarged capillaries (12, 13).

In recent years, biologics have been reported to inhibit the progression to PsA (14). However, the agents that have been reported primarily consist of antibodies targeting TNF-α, IL-12/23p40, and IL-17. The impact of anti-IL-23p19 antibodies remains uncertain. This study aims to examine the therapeutic response and mechanism of action of tildrakizumab, assess its influence on nailfold capillary changes, and evaluate its potential to impede the progression to PsA.

Methods

Patients

Data for this prospective study were collected from patients diagnosed with psoriasis vulgaris, who provided informed consent at the University of Tokyo Hospital, Takahashi clinic, or MisatoKenwa Hospital and MisatoKenwa clinic between January 2020 and April 2023. The study sample consisted of 246 patients with psoriasis vulgaris (PsV) without arthritis. PsA patients were diagnosed by rheumatologists using the CASPAR criteria (15). Only patients who had not previously received any topical treatment for distal interphalangeal (DIP) joints and nails, or any systemic treatment, were included in this study. Exclusion criteria encompassed evidence of vascular disorders, hepatitis, collagen diseases, other skin diseases, infection, and drug abuse. The nailfolds of all fingers were examined for capillaroscopic changes, and the number and distribution of nailfold videocapillaroscopy (NVC) findings in each finger were recorded. This study received approval from the University of Tokyo Ethics Board. Patients or the

public were not involved in the design, conduct, reporting, or dissemination plans of this research.

Observation of the nailfold capillaries

The nailfold capillaries were observed using the TOKU Capillaro-01 device (Toku Co., Tokyo, Japan). NVC examinations were conducted at each patient visit to determine their NVC findings. Prior to the test, patients were instructed to abstain from consuming caffeine for 12 hours. The patients were positioned in a supine position for 15 minutes at a room temperature of 22 to 25°C. Ten nailfolds were examined in each patient. The same dermatologist evaluated capillaroscopic parameters such as nailfold bleeding (NFB) and irregularly enlarged capillaries for each image. The evaluation methodology, items, and software used for the evaluation were consistent with previous descriptions (12).

Isolation of human peripheral blood mononuclear cells and fluorescent antibody staining

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples using HetaSep (Stem Cell Technologies Inc., Vancouver, Canada) through gradient centrifugation. Following Fc blocking (I-4506, Sigma-aldrich, MO, USA), the cells were labeled with PE-labeled CD4 (MHCD0404, Thermo Fisher, Waltham, MA, USA), FITC-labeled CD25 (11-0257-42, Thermo Fisher), PE/Cy7-CD127 (25-1278-42, Thermo Fisher), APC-CD3 (17-0032-82, Thermo Fisher), PE/Cy7-CCR6 (25-1969-42, Thermo Fisher), or VioBright FITC-CXCR3 (130-106-009, Miltenyi-Biotec, Bergisch-Gladbach, Germany). The samples were then incubated at room temperature in the dark for 30 minutes. Subsequently, the samples were washed twice with PBS. After membrane staining, the cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Flow cytometry analysis was performed using a BD FACSVerseTM flow cytometer with BD FACSuiteTM software (BD Biosciences, Germany). All analyses were conducted using fresh blood samples.

T-cell proliferation and co-culture suppression assays

CD4+CD25+ regulatory T cells were isolated following the protocol provided by the manufacturer (130-091-301, Miltenyi-Biotec). The proliferative capacity of the isolated CD4+CD25+ or CD4+CD25- T cells was assessed using the protocol provided by the manufacturer (130-092-909, Miltenyi-Biotec). The suppressor capacity of T cells was investigated through co-culture assays. The proliferative capacity of isolated CD4+CD25+ or CD4+CD25- T cells (5 x 104) was analyzed by bromodeoxyuridine (BrdU), after stimulation with anti-CD3/CD28-coated beads (Invitrogen, Breda, the Netherlands) with or without exogenously added recombinant human IL-2 (12.5 U ml-1). The suppressor capacity of T cells was studied in co-culture assays. In brief, CD4+CD25- (5 x 104) T cells were stimulated with anti-CD3/CD28-coated beads in the absence and presence of decreasing numbers of CD4+CD25+ or CD4+CD25- T cells. Cell proliferation was analyzed at day 4 of the cultures by specific anti-BrdU ELISA (Roche, Meylan, France).

Statistical analysis

To examine the factors associated with predicting the response to PASI or the development of PsA, a logistic regression model or Cox regression analysis was constructed. This analysis included PsA risk factors such as involvement of the scalp, nails, and buttocks, while adjusting for age, sex, psoriasis severity, and body mass index. Logistic regression analysis was employed to identify the key factors that coexisted with PsA, while Cox regression analysis was used to identify the key factors that predicted the development of PsA. The significance of covariate effects was determined using a two-sided Wald's test with a p-value threshold of less than 0.05. Following the univariate analysis, the significant factors were further analyzed in a multivariate analysis. Other statistical significance was assessed using various tests, including the Mann-Whitney U-test, Wilcoxon signed-rank test, student's t-test, paired t-test, $\chi 2$ test, Log-rank test, and Spearman's rank correlation test. All statistical analyses were conducted using JMP Pro 14 software. A p-value of less than 0.05 was considered statistically significant.

Results

More severely affected patients were treated in the tildrakizumab group compared to the topical group

A total of 246 patients diagnosed with Psoriasis Vulgaris (PsV) were enrolled in the present study, as indicated in Table 1. Among

TABLE 1 Characteristics of included patients treated with tildrakizumab or topical treatments.

Baseline	Tildrakizumab (n = 20)	Topical (n = 226)	P-value
Age (years)	63 (20)	54 (20)	0.07
Sex (male/female)	15/5	147/79	0.47
BMI	23.9 (2.9)	22.9 (4.0)	0.17
Skin duration (years)	12 (11)	10 (12)	0.42
PASI	7.5 (8.6)	2.3 (3.1)	<0.0001
Scalp involvement	18 (90.0%)	109 (48.2%)	0.0003
Nail involvement	9 (45.0%)	63 (27.9%)	0.13
Buttock involvement	18(90.0%)	32 (14.2%)	<0.0001
NFB	19 (95.0%)	81 (35.8%)	<0.0001
Enlarged capillaries	19 (95.0%)	59 (26.1%)	<0.0001

Data are n (%) or mean (SD). BMI; body mass index, NFB; nailfold bleeding.

these patients, twenty individuals received treatment with tildrakizumab, while the remaining participants were solely treated with topical agents. It was observed that patients who received tildrakizumab exhibited significantly elevated PASI scores (7.5 ± 8.6 *vs.* 2.3 ± 3.1, $p < 0.0001$), significantly more scalp lesions (18 (90.0%) *vs.* 109 (48.2%), $p = 0.0003$), and significantly more buttock lesions (18 (90.0%) *vs.* 32 (14.2%), $p < 0.0001$), a significantly higher proportion with NFB (19 (95.0%) *vs.* 81 (35.8%), $p < 0.0001$), a significantly higher proportion with enlarged capillaries (19 (95.0%) *vs.* 59 (26.1%), $p < 0.0001$) compared with topical group. Among the risk factors for the development of PsA (16–18), a significantly higher percentage had scalp, buttocks, NFB and enlarged capillaries. It was suggested that those at higher risk of developing PsA were more likely to be treated with tildrakizumab.

Tildrakizumab can achieve better skin lesions in the elderly

Here, age ≥ 65 is defined as "elderly." The number of elderly patients in the tildrakizumab group was 11. The differences in background factors between the two groups of patients are shown in Supplementary Table S1. There were no significant differences other than age. We first attempted to identify the clinical characteristics of the 20 patients treated with tildrakizumab who achieved PASI clear or PASI ≤ 2 at 7 months post-treatment (Table 2). In logistic regression analysis, we compared age, gender, BMI, duration of skin lesions, PASI, scalp, nail, and buttock lesions, NFB, and presence of enlarged capillaries. As a result, age≥65 was the only significant variable in the multivariate analysis. Similarly, we identified clinical characteristics of patients who were able to achieve PASI ≤ 2 (Table 3). Similarly, age≥65 was the only significant variable. In summary, the results suggest that age is an important factor in predicting treatment responsiveness of skin lesions after 7 months of tildrakizumab.

Tildrakizumab improves the number and function of Tregs reduced by aging

The above analysis suggests that age is an important factor in determining response to treatment with tildrakizumab. We focused on Treg as one factor explaining this phenomenon. Since it has been reported that the number of Tregs is decreased and Treg function is impaired in psoriasis and in the elderly (9), we first examined differences in the number of Tregs and T helper 17 (Th17) cells with age in psoriasis patients (Figure 1A). The number of Treg was significantly decreased in the elderly, whereas that of Th17 showed no age-related differences (Figure 1A). Analysis of the reduced Treg function also revealed that the suppressive capacity of Tregs was also reduced (Figure 1B). Therefore, we examined these Tregs and Th17 before and after treatment with tildrakizumab (Figure 1C). Tregs were significantly increased and Th17 was significantly decreased after treatment with Tildrakizumab (Figure 1C). The suppressive function of Treg was also improved by treatment with tildrakizumab (Figure 1D). These findings suggest that one factor explaining the higher efficacy of tildrakizumab in the elderly may be its ability to improve the number and function of reduced Tregs.

Tildrakizumab improves NFB and enlarged capillaries, risk factors for progression to PsA, and inhibits the progression to PsA

Thus, tildrakizumab acts at the cellular level and improves the skin lesions of psoriasis. Th17 cells produce inflammatory cytokines, which have also been implicated in nailfold capillary

TABLE 2 Factors that predict PASI clear after 7 months of tildrakizumab treatment.

	Univariate		Multivariate	
	Likelihood ratio	p-value	Likelihood ratio	p-value
Age ≧ 65 (years)	17.09	<0.0001	16.15	<0.0001
Sex (male)	0.61	0.44	0.76	0.38
BMI ≧25	0.31	0.58		
Skin duration ≧ 10 (years)	0.91	0.34		
PASI ≧10	5.60	0.02	3.26	0.07
Scalp involvement	2.57	0.11		
Nail involvement	0.74	0.39		
Buttock involvement	2.57	0.11		
NFB	1.24	0.27		
Enlarged capillaries	1.24	0.27		

CI, confidence interval; BMI, body mass index; PASI, psoriasis area and severity index; NFB, nailfold bleeding.

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TABLE 3 Factors that predict PASI ≦ 2 after 7 months of tildrakizumab treatment.

	Univariate		Multivariate	
	Likelihood ratio	p-value	Likelihood ratio	p-value
Age ≧ 65 (years)	7.64	0.006	6.16	0.01
Sex (male)	0.00	1.00	0.37	0.54
BMI ≧25	0.21	0.65		
Skin duration ≧ 10 (years)	1.86	0.17		
PASI ≧10	2.41	0.12	1.24	0.26
Scalp involvement	0.95	0.33		
Nail involvement	0.05	0.82		
Buttock involvement	0.95	0.33		
NFB	0.46	0.50		
Enlarged capillaries	0.46	0.50		

CI, confidence interval; BMI, body mass index; PASI, psoriasis area and severity index; NFB, nailfold bleeding.

abnormalities (12). In fact, it was discovered that treatment with tildrakizumab resulted in significant improvements in NFB and enlarged capillaries, both of which are risk factors for the development of PsA. These improvements were observed as early as one month after treatment initiation and were sustained throughout the study period (Figure 1E). During the course of the study, 36 patients in the topical group and 1 patient in the tildrakizumab group developed PsA. A multivariate analysis using a Cox proportional hazards model identified age, nail lesions, NFB, and enlarged capillaries as risk factors for the progression to PsA. However, treatment with tildrakizumab was found to be effective in preventing the progression to PsA (Table 4). In conclusion, these findings demonstrate that tildrakizumab not only alleviates psoriasis at the cellular level but also improves NFB and enlarged

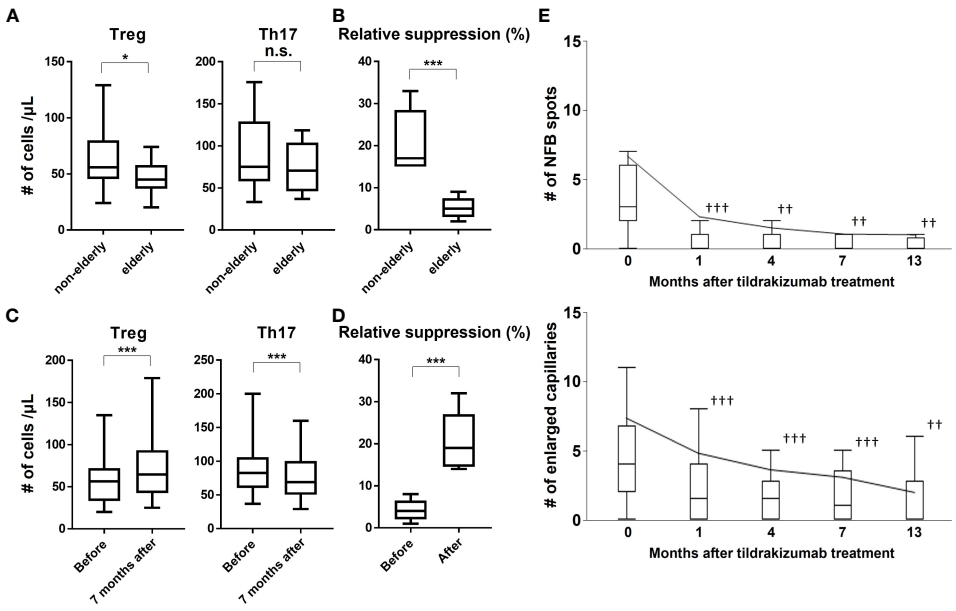

FIGURE 1
Treg number and function are reduced in the elderly, and tildrakizumab improves them and also NFB and enlarged capillaries, risk factors for progression to PsA. We examined the numbers of Treg and Th17 in the peripheral blood of elderly and non-elderly subjects **(A)** and their suppressive ability **(B)**. We also examined the change in the number of Tregs and Th17 **(C)** and their suppressive capacity before and after Tildrakizumab treatment **(D)**. The number of NFBs and enlarged capillaries were examined before and at 1, 4, 7, and 13 months after treatment with tildrakizumab **(E)**. Box plot shows median, 25th, and 75th percentile, and whiskers show the standard deviation. *p<0.05, ***p<0.005. ††p<0.01, †††p<0.005 *vs.* before treatment. n.s. = not significant.

10.3389/fimmu.2023.1286251

TABLE 4 Factors that predict or prevent the development of PsA.

	Univariate			Multivariate with NFB			Multivariate with enlarged capillaries		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Age (/1year)	1.02	1.00-1.04	0.06	1.02	1.00-1.04	0.02	1.02	1.00-1.04	0.02
Sex (Female)	0.99	0.49-1.97	0.97	1.51	0.73-3.13	0.26	1.36	0.67-2.75	0.39
BMI									
≧25 *vs* 25>	1.00	0.50-2.03	0.99						
PASI									
≧10 *vs* 10>	0.79	0.19-3.31	0.75						
Scalp involvement	1.28	0.66-2.48	0.47						
Nail involvement	2.85	1.49-5.46	0.002	3.10	1.60-6.02	0.0008	2.44	1.23-4.82	0.01
Buttock involvement	1.01	0.47-2.20	0.97						
NFB	2.15	1.11-4.15	0.023	2.92	1.44-5.93	0.003			
Enlarged capillaries	3.44	1.78-6.66	0.0002				4.61	2.27-9.38	<0.0001
Tildrakizumab	0.17	0.02-1.28	0.09	0.06	0.007-0.46	0.007	0.04	0.01-0.34	0.003

CI, confidence interval; BMI, body mass index; PASI, psoriasis area and severity index; NFB, nailfold bleeding.

capillaries, which are known risk factors for the development of PsA, ultimately inhibiting the progression to PsA.

Discussion

In this study, a significantly higher percentage of patients in the tildrakizumab group had scalp, buttocks, NFB and enlarged capillaries, and those at higher risk of progressing to PsA were treated with more tildrakizumab compared to the topical group (Table 1). Among those treated with tildrakizumab, those who achieved better skin lesions at 7 months post-treatment were older (Tables 2 and 3). One factor that may explain this is the importance of Tregs, which were found to be more reduced and less functional in elderly patients with psoriasis (Figures 1A, B). These abnormalities improved with treatment with tildrakizumab (Figures 1C, D). One factor that may explain the higher efficacy of tildrakizumab in elderly patients is that it may be more effective in improving the number and function of reduced Tregs. Tildrakizumab also improved NFB and enlarged capillaries, one of the risk factors for progression to PsA (Figure 1E), and inhibited progression to PsA (Table 4). These results suggest that tildrakizumab improved psoriasis at the cellular level, ameliorated NFB and enlarged capillaries, one of the risk factors for progression to PsA, and inhibited progression to PsA.

To date, factors determining response to treatment with tildrakizumab have not been identified. For the first time in this study, tildrakizumab restores the number and function of Tregs and is more effective, especially in the elderly. It has been reported that Treg function is reduced in the elderly (9). Some researchers have reported a decreased percentage of Tregs in peripheral blood of psoriasis patients (19–21), while others showed no difference in circulating Treg frequency (22–25). However, differences in Tregs by age have not been investigated in patients with psoriasis. This study suggested that Tregs may be reduced in number and function especially in the elderly psoriasis patients. Tildrakizumab, an anti-IL-23 antibody that targets IL-23, which is known to act on Tregs, as a therapeutic target, was thought to be more effective in the elderly by targeting them.

PsA is a disease in which immune abnormalities are strongly implicated; it has been suggested that in the development to PsA, the disease progresses to skin lesions, immune abnormalities, subclinical and clinical PsA (26). Tildrakizumab, an anti-IL-23 antibody, acts at the cellular level and is thought to improve the skin lesions of psoriasis by inhibiting the differentiation and proliferation of Th17 cells and the pathogenic conversion of Tregs to Th17 cells (27). IL-23 produced by macrophages, dendritic cells, and B cells (28) can act on Tregs, making them Th17-like and exacerbating the skin lesions of psoriasis (27). Treg functions include reducing inflammation and autoimmunity. In recent years, a number of reports have emerged that the use of biologics for skin lesions can inhibit their progression to PsA (14). However, only the types of antibodies were reported, such as anti-TNF-α, anti-IL-12/23p40, anti-IL-17, and anti-IL-23p19 antibodies, and there are few reports on whether individual antibodies inhibit progression to PsA. This study shows for the first time that tildrakizumab improves NFB and enlarged capillaries, which are risk factors for PsA, and reduces progression to PsA.

Capillary abnormalities such as NFB and enlarged capillaries are thought to be secondary to the systemic inflammation of psoriasis (12). Indeed, the correlation between serum cytokine levels and capillary abnormalities may support these views. Therefore, it is reasonable that tildrakizumab, which targets IL-23, an inflammatory cytokine, ameliorated nailfold capillary

abnormalities. A strong association between inflammatory cytokines and capillary abnormalities is suggested by the reported efficacy of brodalumab, an anti-IL-17RA antibody, in the treatment of systemic sclerosis, one of the most common diseases showing nailfold capillary abnormalities (29–31).

Taken together, tildrakizumab is particularly effective in the elderly. Its high safety profile also makes it safe for use on the elderly (32). The mechanism of action was suggested to be targeting Tregs. Improvement of risk factors for progression to PsA and, indeed, inhibition of progression to PsA were revealed. The primary drawback of this study is its exclusive focus on Japanese patients. The potential influence of genetic variations between the Japanese population and other ethnic groups on treatment response cannot be overlooked. Another limitation of this study is the low patient number. To ascertain the efficacy of tildrakizumab in preventing PsA in psoriasis, it is anticipated that future large-scale clinical trials will encompass diverse ethnic populations.

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 humans were approved by the ethics committee of the University of Tokyo Graduate School of Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

TF: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. TY: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AE: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. YN: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. StT: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AY: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SkT: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. HK: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. KM: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SS: Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software. AY: Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank Maiko Enomoto for technical support.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1286251/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 26 October 2023
DOI 10.3389/fimmu.2023.1290740

OPEN ACCESS

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RECEIVED 08 September 2023
ACCEPTED 09 October 2023
PUBLISHED 26 October 2023

CITATION
Vollmer C, Dias A, Sales M, Sacramento PM, Silva JC, Oyamada HAA, Linhares UC, Gupta S, Kasahara TM and Bento CAM (2023) Leptin favors imbalance of antigen-specific CD4$^+$ T-cells associated with severity of cat allergy. *Front. Immunol.* 14:1290740. doi: 10.3389/fimmu.2023.1290740

Leptin favors imbalance of antigen-specific CD4$^+$ T-cells associated with severity of cat allergy

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Introduction: Obesity can complicate IgE-mediated allergic diseases. In the present study, we aimed to investigate the ability of obesity-related concentrations of leptin to modulate the *in vitro* effector and regulatory Fel d1-specific CD4$^+$ T-cell subsets in patients allergic to cat, considered the third most common cause of respiratory allergy in humans.

Methods: For this study, plasma and peripheral blood mononuclear cells (PBMC) from 30 cat-allergic patients with mild, moderate and severe respiratory symptoms were obtained. The PBMC cultures were stimulated with Fel d1 antigen (10 μg/mL) in the presence or absence of obesity-related leptin dose (50 ηg/mL). After 6 days, the levels of cytokines and IgE in the supernatants were evaluated by multiplex and ELISA, respectively. The frequency of different non-follicular (CXCR5$^-$) and follicular (CXCR5$^+$) Fel d1-specific CD4$^+$ T cell subsets was determined by flow cytometry. The plasma levels of leptin and IgE anti-cat titers were evaluated by ELISA and ImmunoCAP, respectively.

Results and conclusions: Fel d1 induced both IgE production and release of cytokines related to Th2, Th9 and Th17 cell phenotypes. Feld1 was more efficient in increasing the frequency of T_{FH}IL-21$^-$ cells positive for IL-4, IL-5 and IL-13 than T_{FH}IL-21$^+$ cell subsets. Leptin favored the expansion Th2-like and Th9-like cells and T_{FH}IL-21$^-$ cells positive for IL-4, IL-5 and IL-13, but reduced the proportion of conventional (Treg/Tr-1) and follicular (T_{FR}) regulatory CD4$^+$ T-cell subsets expressing or not CD39 marker. Finally, many of the imbalances between Fel d1-specific CD4$^+$ T-cells were also correlated with plasma leptin and anti-Fel d1 IgE titers. In summary, hyperleptinemia should negatively impact on the severity of cat allergies by favoring the expansion of pathogenic Fel d1-specific CD4$^+$ T-cell phenotypes and damaging the functional status of regulatory CD4$^+$ T-cell subsets.

KEYWORDS

leptin, Fel d1, Th2/Th9, Tfh cells, Treg/Tr-1 cells, Tfr cells

1 Introduction

Cat allergies are the most common mammalian-origin allergy in humans, affecting approximately 1 in 5 adults worldwide (1, 2). The most common clinical presentations in these patients are rhinitis, asthma, and/or conjunctivitis. When persistent, the clinical symptoms may impair quality of life (3, 4). Furthermore, severely allergic patients may present an anaphylactic reaction, requiring emergency medical care. Although eight allergens derived from cats have been described, designated Fel d1 to d8, only Fel d1 has clinical significance, accounting for up to 96% of allergic sensitization to cats in humans (5, 6). Primarily produced by salivary and sebaceous glands (5). Fel d1 can easily become and remain airborne in dander and dust particles for extended periods (5, 6).

The hallmark of cat sensitization and symptom severity is the production of high-affinity Fel d1-specific IgE (6, 7). Although Th2 cytokines, IL-4 and IL-13, can increase IgE production, the synthesis of this antibody is critically dependent on B cell collaboration with follicular helper T (T_{FH}) cells (8). T_{FH} cells are specialized $CD4^+$ T cells that provide help to B cells activation into germinal center (GC) of lymphoid follicles. In the GC, T_{FH} cells are characterized by high expression of CXCR5, programmed cell death protein (PD-1), B cell lymphoma 6 (Bcl-6), and IL-21 production (9). The main function of CXCR5 is to guide T_{FH} cells migration towards lymphoid follicles in response to its ligand, the CXCL13, abundantly produced by GC-derived B cells (9). On the other hand, IL-21 from T_{FH} cells not only mediate the selection of high-affinity and isotype switched B cells, but also promote differentiation of these lymphocytes into plasma cells and memory B cells (9). Although T_{FH} cells in peripheral blood are Bcl-6 negative and express low PD-1 levels, they are able to induce antibody production from peripheral B cells (9). Based on cytokines, human circulating T_{FH} cells have been classified as $T_{FH}1$ ($IL\text{-}21^+IFN\text{-}\gamma^+$), $T_{FH}2$ ($IL\text{-}21^+IL\text{-}4^+$), $T_{FH}17$ ($IL\text{-}21^+IL\text{-}17^+$) and, more recently, $T_{FH}13$ ($IL\text{-}21^{low}L\text{-}4^{hi}IL\text{-}5^{hi}IL\text{-}13^{hi}$) (8).

Many studies have demonstrated the involvement of $T_{FH}2$ cells in the pathogenesis of allergic IgE-mediated airway diseases (10–16). In patients suffering from allergic rhinitis and asthma, elevated frequency of circulating $T_{FH}2$ has been associated with plasma IgE titers and clinical exacerbation (10–14). Moreover, the expansion of $T_{FH}2$ cells inside the airways of allergic patients appear to promote IgE production by local activated B cells, which may play an important role in mast cells and eosinophil activation (15, 16).

Interestingly, more recent studies have demonstrated that, while $T_{FH}2$ cells induce low-affinity IgE production, the synthesis of high-affinity IgE to allergens critically depends on $T_{FH}13$ cells (8, 17). The binding of high-affinity IgE/allergen to FcεRI on mast cells and basophils immediately triggers histamine release, quickly causing a cluster of typical cat allergic symptoms (18). Further, in addition to mast cells, eosinophils activated by the same Fel d1/IgE complexes contribute to allergy pathogenesis by producing lager amounts of leukotrienes (LTC4, LTD4, and LTE4) and platelet-activating factor (PAF), pro-inflammatory lipids that induce local vasodilatation, edema, neurogenic stimulation, smooth muscle contraction and hypersecretion of mucus (19). Moreover, IL-9-secreting $CD4^+$ T (Th9) cells have also been implicated in atopic allergy (20). IL-9 prolongs the survival of mast cells, potentiates IgE production and amplifies the ability of IL-5 and IL-13 to increase eosinophil survival and mucus production (20).

As well as inducing the $Th2/T_{FH}2/T_{FH}13$ axis, the severity of IgE-mediated allergies has been associated with functional impairment of non-follicular [Treg ($CXR5^-FoxP3^+IL\text{-}10^+$) and Tr-1 ($CXCR5^-FoxP3^-IL\text{-}10^+$)] and follicular [$T_{FR}$ ($CXCR5^+$ $FoxP3^+IL\text{-}10^+$)] regulatory $CD4^+$ T cells (21, 22). While T_{FR} cells control IgE production by B cells in GCs, Treg and Tr-1 cells are essential to reducing inflammatory cytokine release by local mast cells, eosinophils and Th2 cells (21, 22). Therefore, any adverse event that favors $Th2/T_{FH}$ cell expansion and damages regulatory $CD4^+$ T cell phenotypes should affect the severity of atopic diseases, such as obesity.

Obesity has been related to severity of allergy symptoms and to higher levels of total and allergen-specific IgE in atopic individuals (23, 24). In cat allergic patients, obesity was associated with total and allergen-specific IgE levels (25). This adverse relationship must be, at least partially, associated with high leptin production, an adipokine known to modulate the functional status of T cells (26).

Leptin is a 16 kDa peptide encoded by the OB gene. At physiological concentrations, leptin plays an adjuvant role in the immune response against different pathogens (27). However, hyperleptinemia, as observed in obese individuals, has been correlated with the severity of allergic reactions (28). Ciprandi et al. (29) demonstrated a direct relationship between IgE titers and eosinophil counts with leptin levels in patients with allergic rhinitis. With regard to $CD4^+$ T cell phenotypes, studies published by our group performed in patients with allergic asthma (AA) have found a positive correlation between plasma leptin levels and circulating Th2- and Th17-like cells able to produce high levels of IL-5, IL-6 and IL-17 in response to mitogen (30, 31). In addition, the frequency of these pro-inflammatory $CD4^+$ T cell subsets were directly associated with lung function impairment (31). Still according to our previous study, in $CD4^+$ T cell cultures from lean AA patients, obesity-related leptin concentration enhanced Th2- and Th17-related cytokine production and impaired Treg function in response to polyclonal activators (31). However, studies regarding the effects of leptin on the composition of different allergen-specific $CD4^+$ T-cells have not been conducted to date. Therefore, the main objective of the present study was to investigate the ability of obesity-related leptin doses to modulate the *in vitro* different effector and regulatory Fel d1-specific $CD4^+$ T cells from patients with persistent cat allergies.

2 Materials and methods

2.1 Subjects

Thirty patients with allergic rhinitis (AR) and/or asthma (AA) to cat dander were recruited from March 2020 to September 2021 from

Abbreviations: Allergic asthma (AA), allergic rhinitis (AR), B cell lymphoma 6 (Bcl-6). body mass index (BMI), germinal center (GC), healthy subjects (HS), Leptin (Lep), monoclonal antibodies (mAbs), non-follicular helper T (non-T_{FH}), peripheral blood mononuclear cells (PBMC), programmed cell death protein-1 (PD-1), follicular helper T (T_{FH}), follicular regulatory T cells (T_{FR}).

the Federal University of the State of Rio de Janeiro Hospital/UNIRIO (Rio de Janeiro, Brazil). All patients had a skin-prick test and IgE positive for cat dander extract (Table 1). Since AA is a disorder characterized by inflammation of the airways and recurrent episode of breathing difficulties triggered by allergens, among our patients, persistent AA was diagnosed by a history of recurrent wheezing, dyspnea and chest tightness, and confirmed by methacholine bronchial hyperresponsiveness, when FEV1 was ≥ 70%, or bronchial reversibility after salbutamol inhalation (when FEV1 was <70%). According to daily frequency, severity of clinical exacerbation, lung function damage and need to hospital admission, AA is classified as mild, moderate or severe (21). Also, interference in daily activities is also taken into account (21). With regard to AR, symptom severity was determined by using the total nasal symptom score (TNSS), which is calculated as the sum of scores for each of nasal congestion, sneezing, nasal itching, and rhinorrhea at each time point, using a four point scale (0–3), where 0 indicates no symptoms, 1 for mild symptoms, 2 for awareness of symptoms (but tolerable), and score 3 for severe symptoms that are hard to tolerate and interfere with daily activity (32) (Table 1). We excluded patients taking oral or intravenous steroids, theophylline, long-acting β2-agonists, leukotriene antagonists or antihistamines 1 month prior to the study. As control group, twenty healthy subjects (HS), matched for age and sex and with no history of allergic diseases, were also recruited into the study. According to the body mass index (BMI), subjects were stratified as lean (BMI from 18.5 to 24.9), overweight (BMI from 25 to 29.9) and class I obesity (BMI from 30 to 35). Regardless of experimental group, smoking individuals and those with history of upper or lower airway infectious disease 2 months prior to recruitment were also excluded of the study. The Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (CAAE 44951215.6.0000.5258), approved the study, and blood was collected only after written informed consent was obtained from each individual.

2.2 Cell cultures

Peripheral blood was collected in heparin-containing tubes (BD Vacutainer, Franklin Lakes, NY) and peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on the Ficoll–Hypaque density gradient. Fresh viable PBMCs (1×10^6/mL) were cultured in 24-well flat-bottomed microplates with 2 mL of RPMI medium (ThermoFisher Scientific Inc.) supplemented with 2 μM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% fetal calf serum, 20 U/mL of penicillin, 20 μg/mL of streptomycin and 20 mM of HEPES buffer. As positive control, PBMC cultures were stimulated with phytohaemaglutinine (PHA, 1 μg/mL) (Sigma-Aldrich Co) for 3 days in a humidified 5% CO_2 incubator. In order to evaluate the antigen-specific response, the cells were stimulated with Fel d1 (10 μg/mL) (MyBioSource, San Diego, CA, USA) for 6 days. This concentration of Fel d1 was chosen from a previous study that evaluated T cell response to this antigen (6). In these cultures, the role of leptin (Sigma-Aldrich Co) was determined after the addition of 50 ng/mL of this adipokine. This leptin concentration was determined after a dose-response curve (10, 50 and 100 ng/mL) of cytokine-secreting $CD4^+$ T cells from healthy subjects (HS) and cat-allergic patients (CAP) in PBMC cultures activated with PHA (1 μg/mL) (Sigma-Aldrich Co) (Figure S1). Notably, this leptin concentration is related to the levels of this adipokine in obese subjects (33). After 6 days of culturing, the supernatants were collected, frozen at -20°C for further analysis of cytokine production (Luminex) and IgE levels (ELISA). The PBMC

TABLE 1 The characteristics of subjects.

	Control[1]	CAP[2]		
		Rhinitis	*Asthma*	*Asthma and rhinitis*
N[0] of subjects (n)	20	8	6	16
Gender (female/male) (n)	15/5	6/2	4/2	10/6
Age [(years), mean ± SD]	29.1 ± 13.8	28.8 ± 7.9	31.2 ± 10.1	30.3 ± 8.7
Clinical presentation (n)[3]				
Mild	ND	1	2	1
Moderate	ND	4	3	7
Severe	ND	3	1	8
BMI (n)[4]				
Lean	5	2	2	3
Overweight	10	3	3	6
Obese class I	5	3	1	7

[1]Healthy subjects. [2]Cat allergic patients suffering from rhinitis, asthma alone or rhinitis and asthma to cat dander. [3]The severity of rhinitis and asthma symptom was determined by TNSS (total nasal symptom score) and GINA (Global Initiative for Asthma) criteria, respectively. [4]Body mass index: a value derived from the mass (weight in Kg) and height (in meters) of an individual (lean: 18.5-24.9, overweight: 25-29.9 and obese class I: 30-35). ND, no determined.

was also used to identify different $CD4^+$ T cell phenotypes using flow cytometry.

2.3 Flow cytometry analysis

Different $CD4^+$ T cell subsets in response to Fel d1 were identified by staining the PBMCs with mouse anti-human monoclonal antibodies (mAbs) for CD3-APC-H7 (SK7 clone), CD4-BV605 (T4 clone), CXCR5-PerCP.eF710 (mu5ubee clone), PD-1-APC (MIH4 clone), CD39-FITC (TU66 clone), FoxP3-PECy5.5 (PGH101 clone), IL-4-PECy7 (8D48 clone), IL-5-eFluor450 (TRFK5 clone), IL-9-BV4211 (MH9A3 clone), IL-10-BV722 (JES3-9D7 clone), IL-13-APC (JES10-5A2 clone), IL-17-AF488 (eBio64DEC17 clone) and IL-21-PE (3A3-N2.1 clone). These mAbs and all isotype control antibodies were purchased from Thermo Fischer (San Diego, CA, USA). Briefly, PBMCs were incubated with various combinations of mAbs for surface markers (CD3, CD4, CXCR5, PD-1, and CD39) for 30 min at room temperature in the dark, according to manufacturer's instructions. The cells were washed with PBS + 2%FBS, then submitted to permeabilization by incubating the PBMCs with Cytofix/Cytoperm solution (BD Pharmigen, San Diego, CA) at 4°C for 20 min. After washing, the mAbs for intracellular staining (FoxP3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, and IL-21) were added in different combinations and incubated for 30 min at 4°C. The stained cells were acquired on Attune NxT flow cytometers (Thermo Fisher Corporation) and analyzed using FlowJo (Tree Star, Inc). Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 200,000 to 300,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells, by using propidium iodide, and doublets.

2.4 Luminex, ImmunoCAP and ELISA assays

The titers of plasma IgE anti-cat was determined by florescence enzyme immunoassay with capsulated cellular polymer solid-phase (ImmunoCAP) coupled with cat dander (REF 14.451201, Therm Fischer Sicentific Inc.) with the detection limit ranging from 0.1 to 100 Ku/L. The cut-off value for IgE positivity was considered 0.35 Ku/L. Circulating leptin levels were measured using a commercial ELISA kit following manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). Plates were read at 450 nm in ELISA reader (Dynex Technologies, USA). Lyophilized leptin ranging from 31.3-2000 pg/mL was used to construct the standard curve. The levels of different cytokines and IgE in the supernatants from cell cultures were determined using the "Th1/Th2/Th9/Th17 Cytokine 18-plex human Panel" kit (InvitroGen, San Diego, CA, USA) and human IgE ELISA kit (88-50610-22) (Invitrogen, Thermo Fisher Scientific Co), respectively.

2.5 Statistical analyses

All statistical analyses were conducted using the Prism 8.0 program (GraphPad Software). Comparisons between immune assays in non-stimulated (none) or activated PBMC cultures with Fel d1 and Fel d1/leptin were performed with one-way ANOVA, followed by Tukey test for data with Gaussian distribution, and by Kruskal-Wallis, followed by Dunn's test for data without Gaussian distribution. The nonparametric Mann-Whitney U test and Student's t test were applied to determine whether the two groups were statistically different for nonparametric and parametric variables, respectively. Pearson's and Spearman's correlation were applied for variables with or without normal distribution, respectively. Significance for all experiments was defined as $p<0.05$.

3 Results

3.1 Leptin modulates cytokine and IgE production by Fel d1-stimulated PBMCs from cat-allergic patients

Table 1 shows that most cat allergic patients were overweight/obese women who presented moderate or severe symptoms of rhinitis (AR) and asthma (AA). As no statistical difference among patients with different clinical symptoms (AR x AA x AR/AA) was observed with regard to immunological assays, they were all included together as a single patient group (CAP- cat allergic patients). For the control group, some experiments were additionally performed in 20 age- and gender-matched healthy subjects (HS). Higher levels of IL-4, IL-5, IL-6, IL-13 and IL-17 were observed in CAP-derived PBMC cultures containing plolyclonlly-activated T cells, as compared with HS (Figure S2). In those cell cultures, leptin elevated the release of IL-6, IFN-γ and IL-17 in HS group and the secretion of IL-5, IL-6, IL-13 and IL-17 in CAP group. In contrast, this adipokine reduced the levels of IL-10 secreted by mitogen-activated T cells from both experimental groups (Figure S2). Concerning the cytokine profile in response to Fel d1, this major cat antigen induced not only the production of IL-4, IL-5, IL-13, IL-9, IL-6, IL-17, IL-21 and IL-10 (Figure 1A), but also the secretion of IgE (Figure 1B). The addition of leptin increased the release of IL-5, IL-13, IL-6, IL-17, IL-21 (Figure 1A) and IgE (Figure 1B), but reduced IL-10 production (Figure 1A). Of note, neither medium nor leptin alone were not able to induce detectable cytokine (data not shown). Concerning the control group, Fel d1 only significantly elevated the production of IL-10, with no difference after leptin addition (Figure S3). In patients, *in vitro* IgE production directly correlated with both IL-4 and IL-5, released by Fel d1-stimulated cells (Table 2), and IL-4, IL-5 and IL-9, secreted by Fel d1/Lep-activated PBMC cultures (Table 2). In contrast, in Fel d1/Lep-stimulated cells, IL-10 secretion inversely correlated to IgE (Table 2).

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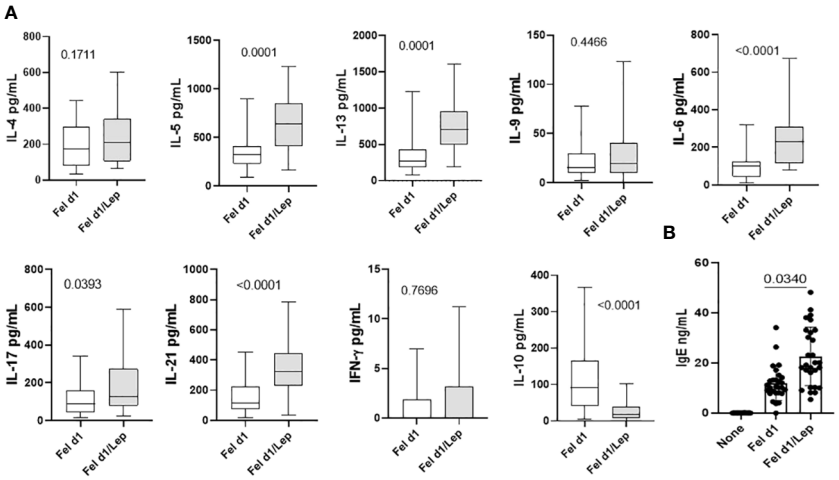

FIGURE 1
Leptin modulates the cytokine profile and IgE production by PBMCs from cat-allergic patients in response to Fel 1d. PBMC cultures (1 x 10^6/mL) from cat-allergic patients (n=30) were maintained for 6 days in the presence of culture medium alone (without) or with 10 µg/mL of Fel 1d, with or without 50 ng/mL of leptin (Lep). At the end of the culture time, the supernatants were harvested and the **(A)** cytokine (IL-4, IL-5, IL-13, IL-6, IL-17, IL-21, IFN-γ and IL-10) and **(B)** IgE levels were determined by Luminex and ELISA, respectively. Mean values were compared using one-way ANOVA and *p* values are shown in the graphs. All data are shown as mean ± SD of six independent experiments with 4 and 6 samples per experiment.

TABLE 2 Correlation between *in vitro* total IgE production and cytokine profile in Fel d1-stimulated PBMC cultures from cat-allergic patients.

	IgE (ng/mL)			
	Fel d1		Fel d1/Lep	
Cytokines (pg/mL)	*r*	*p (n=30)*	*r*	*p (n=30)*
IL-4	0.7324	**0.0002**	0.5343	**0.0152**
IL-5	0.7715	**0.0001**	0.4618	**0.0404**
IL-6	0.1533	0.5187	0.1026	0.6610
IL-9	0.2980	0.2020	0.5648	**0.0095**
IL-13	0.4045	0.0769	0.3488	0.1320
IL-17	0.3263	0.1603	0.2286	0.3323
IL-21	0.1064	0.6553	0.2211	0.3488
IL-10	-0.3143	0.1772	-0.4547	**0.0440**
Non-T_{FH} cells (%)				
IL-4$^+$	0.3177	0.1017	0.2120	0.2451
IL-5$^+$	0.2809	0.2012	0.3223	0.1013
IL-9$^+$	0.4001	0.1003	0.3673	0.1765
IL-13$^+$	0.3181	0.1091	0.1983	0.3412
IL-17$^+$	0.3019	0.1288	0.2883	0.2651
IL-21$^+$	0.2711	0.2188	0.1577	0.4018
IL-21$^+$$T_{FH}$ cells (%)				
IL-4$^+$	0.4011	0.1122	0.4283	0.0657
IL-5$^+$	0.5891	**0.0073**	0.7848	**0.0001**
IL-9$^+$	0.4571	0.1011	0.4122	0.1617

(Continued)

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TABLE 2 Continued

	IgE (ng/mL)			
	Fel d1		Fel d1/Lep	
Cytokines (pg/mL)	*r*	*p (n=30)*	*r*	*p (n=30)*
IL-13$^+$	0.4109	0.1891	0.2512	0.4041
IL-17$^+$	0.3491	0.2019	0.2947	0.3239
IL-21$^-$T$_{FH}$ cells (%)				
IL-4$^+$	0.6012	**0.0085**	0.6966	**0.0023**
IL-5$^+$	0.5901	**0.0091**	0.6818	**0.0031**
IL-9$^+$	0.4112	0.0981	0.6474	**0.0085**
IL-13$^+$	0.4191	0.1187	0.6928	**0.0021**
IL-17$^+$	0.3191	0.2018	0.2729	0.3654
Treg cells (%)				
IL-10$^+$*FoxP3*$^+$ *CD39*$^-$	- 0.1134	0.6019	- 0.2759	0.3589
IL-10$^+$*FoxP3*$^+$ *CD39*$^+$	-0.2133	0.3891	-0.1653	0.5870
Tr-1 cells (%)				
IL-10$^+$*FoxP3*$^-$ *CD39*$^-$	-0.1411	0.6781	-0.1320	0.6653
IL-10$^+$*FoxP3*$^-$ *CD39*$^+$	-0.3712	0.1891	-0.4097	0.1335
T$_{FR}$ cells (%)				
IL-10$^+$*FoxP3*$^+$ *CD39*$^-$	- 0.5781	**0.0212**	- 0.7135	**0.0011**
IL-10 FoxP3$^+$ *CD39*$^+$	-0.6011	**0.0116**	-0.7552	**0.0003**
IL-10$^+$*FoxP3*$^-$ *CD39*$^-$	-0.3019	0.2711	-0.4009	0.1645
IL-10$^+$*FoxP3*$^-$*CD39*$^+$	-0.1092	0.6012	-0.2944	0.3259

In PBMC cultures from cat-allergic patients (n=30) stimulated with Fel d1 or Fel d1/Lep, the levels of cytokines, determined via Luminex, and the frequency of different effector and regulatory CD4$^+$ T cells subsets, evaluated by cytometry, was correlated with the in vitro total IgE concentration, assayed through ELISA.
Bold values indicate significance ($p<0.05$).

3.2 Leptin alters the frequency of effector and regulatory Ag-specific CD4$^+$ T cell subsets in CAP

From identification of CXCR5 and PD-1 markers on CD4$^+$ T cells, and using the gating strategy shown in Figure 2A, no difference in the percentage of non-T$_{FH}$ cells (CXCR5$^-$) (Figure 2B), whole T$_{FH}$ (Figure 2B) and T$_{FH}$ PD-1$^+$ (Figure 2C) cells was observed in the cell cultures stimulated with Fel d1, with or without leptin. In contrast, taking into account the representative dot-plots shown in Figure 2D, Fel d1 elevated the proportion of Th2-like cells [IL-4$^+$ (Figure 2E), IL-5$^+$ (Figure 2F) and IL-13$^+$ (Figure 2H)] and Th9 (IL-9$^+$) cells (Figure 2G), with no change in the percentage of Th17-like cells (IL-17$^+$ and IL-21$^+$) (Figures 2I, J). Leptin elevated the frequency of Fel d1-specific Th2-like cells [IL-5$^+$ (Figure 2F) and IL-13$^+$ (Figure 2H)] and Th17-like cells [IL-17$^+$ (Figure 2I)]. With regards to the classical T$_{FH}$ cells (CXCR5$^+$IL-21$^+$), and following the gating strategy shown in Figure 2K, Fel d1 upregulated the proportion of T$_{FH}$IL-21$^+$IL-17$^+$ (Figure 2P). Notably, Fel d1 more efficiently upregulated the frequency of T$_{FH}$IL-21$^-$ cells positive for IL-4 (Figure 2L), IL-5 (Figure 2M), IL-9 (Figure 2N), and IL-13 in comparison with T$_{FH}$IL-21$^+$cells (Figure 2O). Leptin not only enhanced the proportion of T$_{FH}$IL-21$^+$ IL-5$^+$ (Figure 2M) and T$_{FH}$IL-21$^+$ IL-9$^+$ (Figure 2N), but also that of T$_{FH}$IL-21$^-$ cells positive for IL-4 (Figure 2L), IL-5 (Figure 2M), and IL-13 (Figure 2O). The ability of leptin to upregulate non-T$_{FH}$ and T$_{FH}$ cell phenotypes was observed in cell cultures from lean, overweight and obese patients (data not shown).

Concerning regulatory T cells, through the expression of FoxP3, IL-10 and CD39 on CD4$^+$ T cells, we determined the impact of leptin on modulating the proportion of Fel d1-specific Treg/Tr-1 cells (Figures 3A–C) and T$_{FR}$ cells (Figures 3D–F). Taking into account the gating strategy shown in Figures 3A, D, Fel d1 increased the proportion of Treg (CXCR5$^-$FoxP3$^+$IL-10$^+$) (Figure 3B) and T$_{FR}$ (CXCR5$^+$FoxP3$^+$IL-10$^+$) (Figure 3E), expressing or not CD39. This allergen also upregulated the frequency of Tr-1 (CXCR5$^-$FoxP3$^-$IL-10$^+$CD39$^-$ and CXCR5$^-$FoxP3$^-$IL-10$^+$CD39$^+$) (Figure 3C) and follicular Tr-1-like cells (CXCR5$^+$FoxP3$^-$IL-10$^+$CD39$^-$ and CXCR5$^+$FoxP3$^-$IL-10$^+$CD39$^+$) (Figure 3F). Regardless of cell subtype, leptin significantly reduced the proportion of IL-10-secreting CD4$^+$ T cell subsets (Figure 3).

In vitro IgE production directly correlated with the percentage of T$_{FH}$IL-21$^+$IL-5$^+$ and T$_{FH}$IL-21$^-$ positive for IL-4 and IL-5 in Fel d1-

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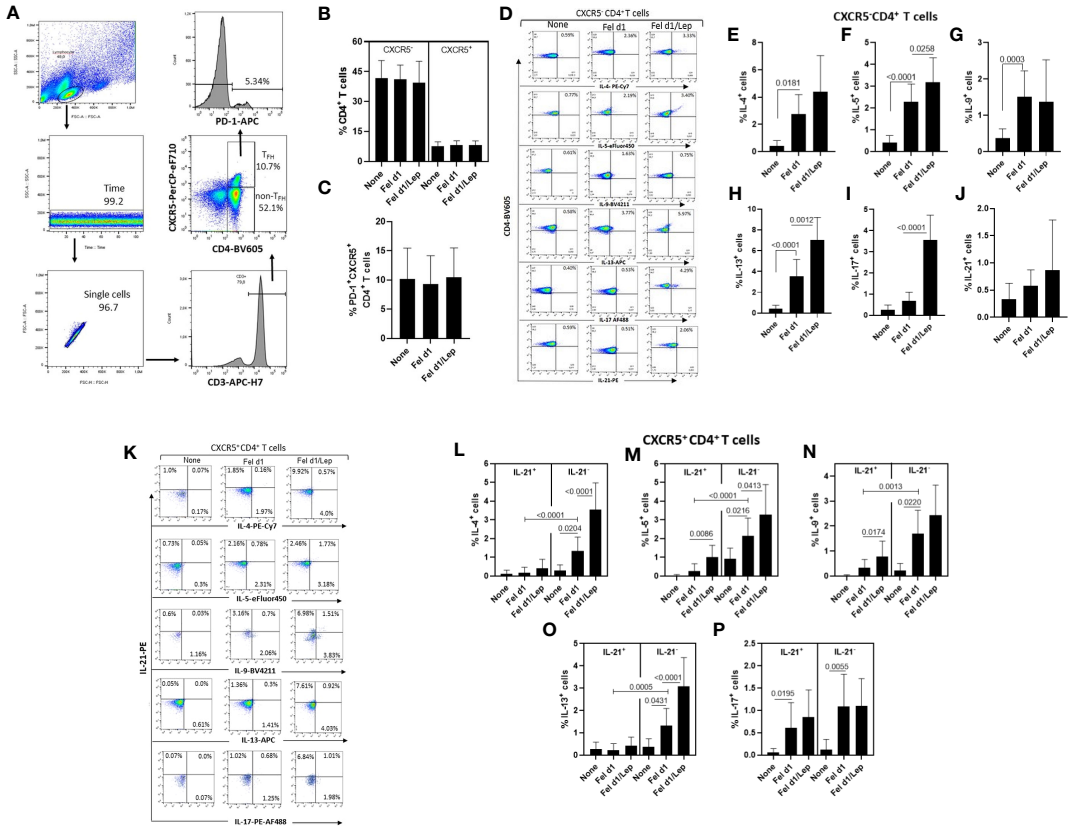

FIGURE 2
Leptin effect on the frequency of different Fel d1-specific T_{FH} and non-T_{FH} cell subsets in cat-allergic patients. PBMCs (1 x 10^6/mL) from cat-allergic patients (n=30) were cultured for 6 days in the presence of culture medium alone (none) or with 10 µg/mL Fel 1d, with or without 50 ng/mL leptin (Lep). At the end of the culture time, and adopting the gating strategy shown in graph **(A)**, the mean ± SD of conventional $CD4^+$ T cells (non-T_{FH}, $CXCR5^-$) and total T_{FH} cells (T_{FH}, CXCR5+) **(B)**, as well as the $T_{FH}PD\text{-}1^+$ cell subset **(C)** were analyzed by cytometry. In **(D, K)**, representative dot-plots of cytokine-producing non-T_{FH} and T_{FH} cells were shown, respectively. In **(E–J)**, the mean ± SD of percentage of **(E)** IL-4^+, **(F)** IL-5^+, **(G)** IL-9^+, **(H)** IL-13^+, **(I)** IL-17^+ and **(J)** IL-21^+ among non-T_{FH} cells, while **(L-P)** showed the mean ± SD values for T_{FH}IL-21^+ and T_{FH}IL-21^- cells able to produce IL-4 **(L)**, IL-5 **(M)**, IL-9 **(N)**, IL-13 **(O)**, and IL-17 **(P)**. Data are shown as mean ± SD of five independent experiments with 2 and 6 samples per experiment. Significance was calculated using one-way ANOVA and *p* values are shown in the graphs.

and Fel d1/Lep-stimulated cell cultures (Table 2). Similarly, higher IgE production was observed in Fel d1/Lep-activated cell cultures with a higher proportion of $T_{FH}IL\text{-}21^-IL\text{-}9^+$ and $IL\text{-}21^-IL\text{-}13^+$ (Table 2). By contrast, IgE negatively correlated with the proportion of allergen-specific $FoxP3^+IL\text{-}10^+$ T_{FR} cells that express, or not, CD39 marker, mainly after leptin addition (Table 2). No relationship was observed for the frequency of allergen-specific non-T_{FH} and Treg cells and IgE levels after leptin addition (Table 2).

Finally, according to BMI, higher frequency of Th2-like cells (IL-5^+ and IL-13^+) (Figure 4A), $T_{FH}IL\text{-}21^+$ (IL-5^+ and IL-17^+) (Figure 4B) and $T_{FH}IL\text{-}21^-$ cell subsets (IL-5^+, IL-9^+ and IL-13^+) (Figure 4C), was observed in obese patients. Conversely, obesity negatively impacted the ability of Fel d1 to elevate Treg (Figure 4D) and T_{FR} cells (Figure 4E), expressing or not CD39, as well as Tr-1 $CD39^+$ cells (Figure 4D).

3.3 Correlation between plasma leptin levels, anti-cat IgE titers and the *in vitro* cytokine profile in CAP.

As demonstrated in Table 3, leptin levels positively correlated with IL-5, IL-6 and IL-17 secretion by Fel d1-stimulated cells, as well as the frequency of both non-T_{FH} (IL-5^+, IL-13^+ and IL-17^+) and $T_{FH}IL\text{-}21^-$ cells positive for IL-5, IL-9 and IL-13. In contrast, a negative correlation was observed between circulating levels of this adipokine and the proportion of Treg and T_{FR} cells, expressing or not CD39 marker. Moreover, the proportion of $CD39^+$Tr-1 cells inversely correlated with leptin concentration (Table 3). Although no significant correlation was observed between plasma leptin and anti-cat IgE (r=0.4054, p=0.0845), titers of this antibody positively correlated with both IL-5 release and the percentage of $T_{FH}IL\text{-}21^-IL\text{-}4^+$ and $IL\text{-}21^-IL\text{-}13^+$ cells in Fel d1-stimulated PBMC cultures (Table 4). On the other hand, higher levels of this antibody were observed in patients with lower Treg and T_{FR} cell proportions, expressing CD39 or not, and Tr-1 $CD39^+$ cells (Table 4).

4 Discussion

Obesity can complicate IgE atopic diseases (23, 34). Here, in cat allergic patients, this adverse relationship should involve, at least in part, increased leptin production that promotes an imbalance between different $CD4^+$ T cell phenotypes specific to Fel d1, the major cat allergen.

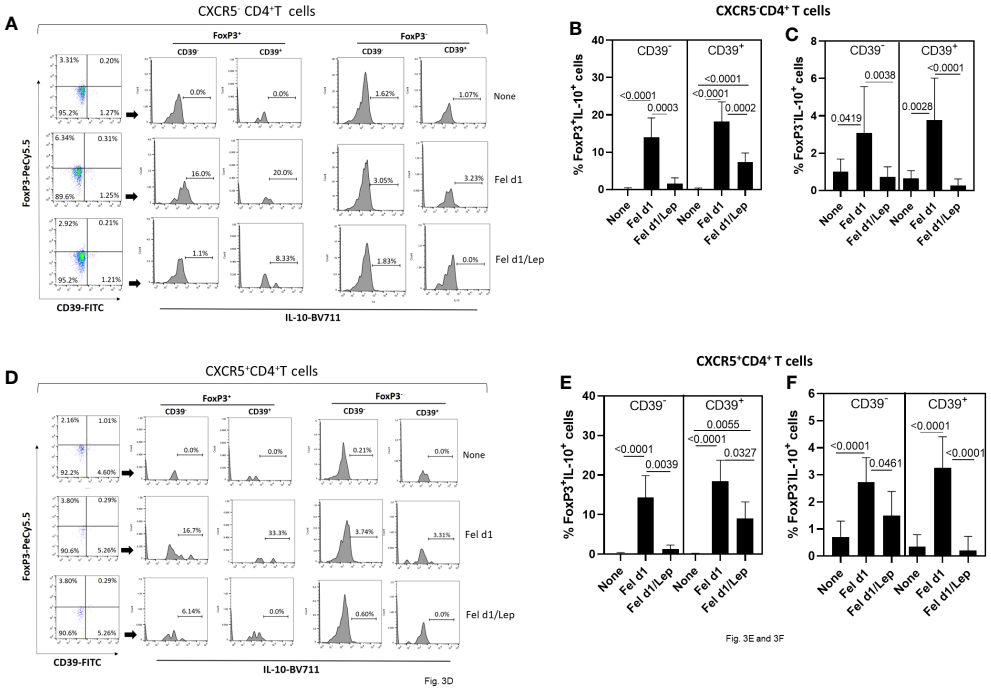

FIGURE 3
Leptin reduced the frequency of Fel d1-specific Tregs and T_{FR} cell subsets in PBMC cultures from cat-allergic patients. Taking into account the expression of CXCR5, FoxP3, IL-10 and CD39, and following representative dot-plots and histograms shown in graphs **(A, D)**, the frequency of **(A)** conventional (Tregs/Tr-1, CXCR5$^-$) and **(D)** follicular (T_{FR}/T_{FR}-1, CXCR5$^+$) regulatory CD4$^+$ T cells was determined in PBMC cultures from cat-allergic patients (n=30) after stimulation for 6 days with Fel d1 and Fel d1/Lep. The mean values (± SD) of FoxP3$^+$IL-10$^+$CD39$^-$ and FoxP3$^+$IL-10$^+$CD39$^+$ **(B, E)**, and FoxP3$^-$IL-10$^+$CD39$^-$ and FoxP3$^-$IL-10$^+$CD39$^+$ **(C, F)**, on Fel D1-specific Treg and T_{FR} cell subsets was determined. Data are shown as mean ± SD of five independent experiments with 2 and 6 samples per experiment. Significance was calculated using one-way ANOVA and *p* values are shown in the graphs.

In the present study, Fel d1 not only increased the production of cytokines related to Th2 and Th9 cells, but also the proportion of antigen-specific T_{FH} cell subsets, mainly IL-21$^-$IL-4$^+$, IL-21$^-$IL-5$^+$ and IL-21$^-$IL-13$^+$. Despite the small sample size in this study, a higher percentage of Fel d1-specific Th2-like cells and T_{FH}2/T_{FH}13 cell phenotypes was observed among obese patients and directly correlated with plasma leptin levels. *In vitro*, this adipokine directly favored the expansion of Fel d1-specific Th2- and Th9-related phenotypes, as well as elevated the percentage of T_{FH}IL-21$^+$ (IL-5$^+$ and IL-9$^+$) and T_{FH}IL-21$^-$ (IL-4$^+$, IL-5$^+$ and IL-13$^+$) cell subsets. Additionally, leptin elevated IgE production. This finding agrees with a study that demonstrated a direct relationship between leptin levels and IgE production in atopic patients (25). Regarding cell phenotypes, IgE levels in PBMC cultures stimulated with Fel d1/Lep directly correlated with the frequency of T_{FH}IL-21$^+$IL-5$^+$ and T_{HF}IL-21$^-$ negative for IL-4, IL-5, IL-13 and IL-9, but not Th2-like cells. Furthermore, plasma titers of anti-Fel d1 IgE positively correlated with T_{FH}IL-21$^-$ positive for IL-5 and IL-13. This finding agrees with studies that demonstrate that T_{FH} cells, but not Th2 cells, are critical for IgE production (8, 17). Among T_{FH} cells, while the T_{FH}2 cell subset induces the production of low-affinity IgE (8). the T_{FH}13 cell subset is responsible for producing high-affinity IgE (17). T_{FH}13 cells are characterized by high IL-4, IL-5 and IL-13 expression associated with very low IL-21 production (25). Yang et al. (35) demonstrated that IL-21 inhibits IgE class-switch recombination in human B cells. Although we did not evaluate either T_{FH} cells that simultaneously express IL-4, IL-5 and IL-13, nor IgE affinity, we believe that the ability of leptin to increase the frequency of Fel d1-specific T_{FH}IL- 21$^-$ able to produce Th2-related cytokines is one of the mechanisms that this adipokine uses to intensify cat allergy severity. Indeed, the formation of high affinity IgE : FcεRI complexes on mast cells, by activating Lyn/Syk/LAT-1 axis, promotes intense and immediate histamine release and leukotriene synthesis (18, 35, 36), resulting in associated allergic symptoms, such as airway constriction, increased mucus production, and coughing.

Despite not directly inducing IgE production, Th2 cytokines participate in the pathogenesis of atopic allergic reactions. IL-4 and IL-13 amplify eosinophil and Th2 cell transmigration to the allergen exposure site (37). IL-5 is responsible for increasing eosinophil formation and survival (38). IL-13 increases B cell survival (18) and compromises respiratory function by increasing mucus production in the airways (39). Therefore, leptin ability to increase the frequency of Fel d1-specific Th2-like cells should impact the severity of allergic reactions to cats. Indeed, leptin, by potentiating Th2-mediated response, has been associated with atopic diseases (40). Moreover, here, leptin also favored expansion of Fel d1-specific Th9-like cells.

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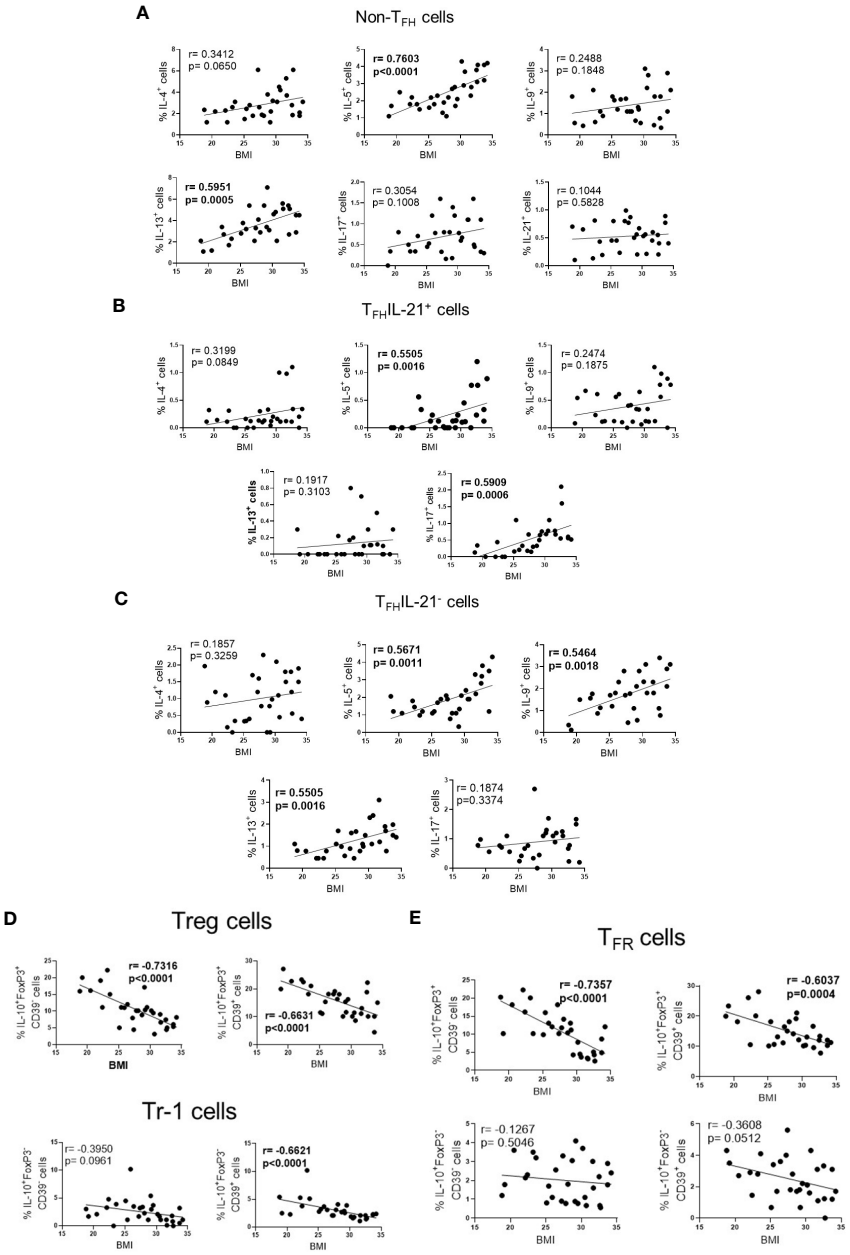

FIGURE 4
The frequency of effector and regulatory Fel d1-specific $CD4^+$ T cell subsets according BMI. In cat-allergic patients (n=30), the frequency of cytokine-producing non-T_{FH} cells **(A)**, IL-21^+ **(B)** and IL-21^- T_{FH} cells **(C)**, as well as Treg/Tr-1 **(D)** and T_{FR} **(E)** cells in response to Fel d1 was correlated with BMI by using Pearson's correlation.

IL-9, along with IL-5 and IL-13, prolong mast cell and eosinophil survival, and increase mucus production (20, 41). Interestingly, despite the lack of data about human $T_{FH}9$ cells, in murine allergy models, these cells support memory IgE^+ B cell generation (42, 43). Finally, the ability of leptin to upregulate Fel d1-induced IL-6 production may also contribute to IgE synthesis, since IL-6 favors B-cell proliferation, plasma cell survival, and antibody production (44–46).

Recently, the severity of mite-allergic asthma has been associated with Der f3-specific Th17 cells (47). Furthermore, IL-17 directly promoted IgE production by human B cells (48) and favors eosinophil accumulation in mucosa of atopic patients (49). In the present study, although Fel d1 has induced $T_{FH}IL\text{-}17^+$, and leptin amplified this cell subtype, no relationship was observed with either *in vitro* IgE production or plasma anti-Fel d1 IgE. However, it is possible that during disease exacerbation, this cell phenotype may

TABLE 3 Correlation between plasma leptin levels and cytokine profile of cells in Fel d1-stimlated PBMC cultures from cat-allergic patients.

	Leptin (ng/mL)	
Cytokines (pg/mL)	*r*	*p (n=30)*
IL-4	0.1998	0.3982
IL-5	0.6438	**0.0022**
IL-6	0.5476	**0.0124**
IL-9	0.1674	0.4805
IL-13	0.3117	0.1810
IL-17	0.4989	**0.0251**
IL-21	0.3013	0.1968
IL-10	-0.2360	0.3165
Non-T_{FH} cells (%)		
IL-4$^+$	0.2822	0.3472
IL-5$^+$	0.7354	**0.0004**
IL-9$^+$	0.2296	0.4475
IL-13$^+$	0.6960	**0.0011**
IL-17$^+$	0.7868	**0.0002**
IL-21$^+$	0.1816	0.5498
IL-21$^+$$T_{FH}$ cells (%)		
IL-4$^+$	0.3877	0.1897
IL-5$^+$	0.3052	0.2096
IL-9$^+$	0.1429	0.6428
IL-13$^+$	0.1447	0.6341
IL-17$^+$	0.3714	0.1605
IL-21$^-$ T_{FH} cells (%)		
IL-4$^+$	0.4014	0.0595
IL-5$^+$	0.6611	**0.0165**
IL-9$^+$	0.7675	**0.0001**
IL-13$^+$	0.8748	**<0.0001**
IL-17$^+$	0.3780	0.1014
Treg cells (%)		
IL-10$^+$*FoxP3*$^+$*CD39*$^-$	-0.8116	**<0.0012**
IL-10$^+$*FoxP3*$^+$*CD39*$^+$	-0.7331	**0.0007**
Tr-1 cells (%)		
IL-10$^+$*FoxP3*$^-$*CD39*$^-$	-04924	0.0893
IL-10$^+$*FoxP3*$^-$*CD39*$^+$	-0.8186	**<0.0001**
T_{FR} cells (%)		
FoxP3$^+$*CD39*$^-$	-0.7015	**0.0024**
FoxP3$^+$*CD39*$^+$	0.7845	**<0.0001**
FoxP3$^-$*CD39*$^-$	0.3011	0.2659
FoxP3$^-$*CD39*$^+$	0.4018	0.0950

(Continued)

TABLE 3 Continued

The levels of cytokines secreted, determined via Luminex, and frequency of different conventional (non-T_{FH}, CXCR5$^-$) and follicular helper (T_{FH}, CXCR5$^+$) CD4$^+$ T cell subsets, evaluated by cytometry, in Fel d1-stimulated PBMC cultures from cat-allergic patients (n=30). were correlated with the plasma leptin concentration, assayed through ELISA. By evaluating the expression of CXCR5, FoxP3 and IL-10, we identified the frequency of non-follicular [Treg (CXR5$^-$FoxP3$^+$IL-10$^+$) and Tr-1 (CXCR5$^-$FoxP3$^-$IL-10$^+$)] and follicular [T_{FR} (CXCR5$^+$ FoxP3$^+$IL-10$^+$)] regulatory CD4$^+$ T cells.
Bold values indicate significance (p<0.05).

TABLE 4 Correlation between total IgE titers and cytokine profiles of Fel d1-stimlated PBMC cultures from cat-allergic patients.

	IgE (Ku/L)	
Cytokines (pg/mL)	*r*	*p (n=30)*
IL-4	*0.2431*	*0.3016*
IL-5	*0.6642*	***0.0014***
IL-6	*0.3848*	*0.0939*
IL-9	*0.1853*	*0.4342*
IL-13	*0.1070*	*0.2160*
IL-17	*0.3757*	*0.1036*
IL-21	*0.3473*	*0.1335*
IL-10	*-0.3304*	*0.1547*
Non-T_{FH} cells (%)		
IL-4$^+$	0.3928	0.1841
IL-5$^+$	0.1107	0.7176
IL-9$^+$	0.3737	0.1736
IL-13$^+$	0.2311	0.4441
IL-17$^+$	0.3407	0.2318
IL-21$^+$	0.3465	0.2671
IL-21$^+$$T_{FH}$ cells (%)		
IL-4$^+$	0.3736	0.2070
IL-5$^+$	0.4101	0.0987
IL-9$^+$	0.1484	0.6298
IL-13$^+$	0.1621	0.5938
IL-17$^+$	0.3301	0.2686
IL-21$^-$$T_{FH}$ cells (%)		
IL-4$^+$	0.6298	**0.0024**
IL-5$^+$	0.4170	0.0601
IL-9$^+$	0.3809	0.1526
IL-13$^+$	0.6823	**0.0078**

(Continued)

TABLE 4 Continued

	IgE (Ku/L)	
Cytokines (pg/mL)	r	p (n=30)
IL-17$^+$	0.3791	0.2025
Non-T_{FH} cells (%)		
IL-10$^+$*FoxP3*$^+$*CD39*$^-$	-0.8473	**<0.0001**
IL-10$^+$*FoxP3*$^+$*CD39*$^+$	- 0.7414	**0.0009**
Tr-1 cells		
IL-10$^+$*FoxP3*$^-$*CD39*$^-$	- 0.3643	0.1017
IL-10$^+$*FoxP3*$^-$*CD39*$^+$	- 0.7986	**0.0001**
T_{FR} cells (%)		
IL-10$^+$*FoxP3*$^+$*CD39*$^-$	-0.7070	**0.0016**
IL-10$^+$*FoxP3*$^+$*CD39*$^+$	-0.8177	**<0.0000**
IL-10$^+$*FoxP3*$^-$*CD39*$^-$	0.1648	0.5089
IL-10$^+$*FoxP3*$^-$*CD39*$^+$	0.1956	0.5189

The titer of plasma total IgE was correlated with both cytokines levels, evaluated by Luminex, and the frequency, determined by cytometry, of different conventional (non-$T_{FH,}$ CXCR5$^-$) and follicular helper (T_{FH}, CXCR5$^+$) CD4$^+$ T cell subsets in Fel d1-stimulated PBMC cultures from cat-allergic patients (n=30).
Bold values indicate significance (p<0.05).

contribute to cat allergy immunopathogenesis by promoting eosinophil infiltration into the airway of patients.

Regarding regulatory CD4$^+$ T cells, the severity of allergic reactions has been associated with functional damage of allergen-specific Treg cells (CXCR5$^-$FoxP3$^+$IL-10$^+$), Tr-1 (CXCR5$^-$FoxP3$^-$IL-10$^+$) and, mainly in IgE-mediated disorders, T_{FR} (CXCR5$^+$FoxP3$^+$IL-10$^+$) (21, 22). In atopic patients, severity of the symptoms correlated with both dysfunctional T_{FR} cells and elevated frequency of $T_{FH}2$ cells highly capable of assisting IgE production by allergen-specific B cells (13, 32, 50, 51). Those atopic-derived T_{FR} cells show impaired IL-10 production, a net anti-inflammatory cytokine (32, 50, 52). In addition to IL-10, several surface biomarkers can identify highly functional regulatory T cells, such as CD39 (53). In the present study, leptin reduced the frequency of Fel d1-specific Treg/Tr-1 and T_{FR} cells, most of them expressing CD39. It is known that CD39, along with CD73, metabolizes the extracellular adenosine triphosphate (ATP)/adenosine diphosphate (ADP) into adenosine (ADO), a metabolite which inhibits pro-inflammatory T cell phenotypes (53). A study by Li et al. observed the role of CD39$^+$Treg cells in controlling airway inflammation in the murine model of allergic asthma (54). Notably, the frequency of Treg and T_{FR} cells, expressing CD39 or not, inversely correlated with both IgE production in Fel d1/Lep-stimulated cell cultures and plasma anti-Fel d1 IgE titers. In agreement with our findings, a study by Martin-Orozco et al. (55), demonstrated an inverse relationship between FoxP3 expression in the regulatory CD4$^+$T cell compartment with serum IgE levels and eosinophilia. Therefore, in the present study, high dose of leptin should negatively impact the prognostic of allergic diseases due to the ability of this adipokine in reducing functional Treg/Tr-1 and T_{FR} cells, CD4 T cell subset implicated in controlling Th2/Th9 and $T_{FH}2$/$T_{FH}13$ axis respectively (21, 22).

5 Conclusions

Although preliminary, our findings suggest that hyperleptinemia, by favoring expansion of pathogenic Fel d1-specific CD4$^+$ T cells and impairing the functioning of regulatory CD4$^+$ T cell subsets, would not only exacerbate disease severity, but also negatively impacts the success of allergen-specific immunotherapies against cat allergies (56, 57).

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 humans were approved by Gaffrée e Guinle university hospital research committee and by the Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (CAAE 44951215.6.0000.5258). The studies were conducted in accordance with the local legislation and institutional requirements. Blood was collected only after written informed consent was obtained from each individual. The participants provided their written informed consent to participate in this study.

Author contributions

CV: Investigation, Methodology, Writing – original draft, Writing – review & editing. AD: Investigation, Methodology, Writing – review & editing. MS: Formal Analysis, Investigation, Methodology, Writing – review & editing. PS: Formal Analysis, Investigation, Methodology, Writing – review & editing. JS: Formal Analysis, Investigation, Writing – review & editing. HO: Investigation, Writing – review & editing. UL: Investigation, Writing – review & editing. SG: Conceptualization, Funding acquisition, Investigation, Writing – review & editing. TK: Conceptualization, Supervision, Writing – review & editing. CB: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article. This study was supported by the Fundação de Amparo à Pesquisa Carlos Chagas Filho (FAPERJ, grant number: E-26/202.940/2017).

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1290740/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 10 November 2023
DOI 10.3389/fimmu.2023.1254176

OPEN ACCESS

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RECEIVED 06 July 2023
ACCEPTED 24 October 2023
PUBLISHED 10 November 2023

CITATION
Rosenberger S, Undeutsch R, Akbarzadeh R, Ohmes J, Enghard P, Riemekasten G and Humrich JY (2023) Regulatory T cells inhibit autoantigen-specific CD4$^+$ T cell responses in lupus-prone NZB/W F1 mice.
Front. Immunol. 14:1254176.
doi: 10.3389/fimmu.2023.1254176

Regulatory T cells inhibit autoantigen-specific CD4$^+$ T cell responses in lupus-prone NZB/W F1 mice

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Introduction: Progressive loss of regulatory T cell (Treg)-mediated control over autoreactive effector T cells contributes to the development of systemic lupus erythematosus (SLE). Accordingly, we hypothesized that Treg may also have the capacity to suppress the activation of autoreactive CD4$^+$ T cells that are considered to drive autoimmunity.

Methods: To investigate whether Treg are involved in the control of autoreactive CD4$^+$ T cells, we depleted CD25$^+$ Treg cells either *in vivo* or *in vitro*, or combined both approaches before antigen-specific stimulation with the SLE-associated autoantigen SmD1(83-119) in the NZB/W F1 mouse model either after immunization against SmD1(83-119) or during spontaneous disease development. Frequencies of autoantigen-specific CD4$^+$ T cells were determined by flow cytometry using the activation marker CD154.

Results: Both *in vitro* and *in vivo* depletion of CD25$^+$ Treg, respectively, increased the frequencies of detectable autoantigen-specific CD4$^+$ T cells by approximately 50%. Notably, the combined *in vivo* and *in vitro* depletion of CD25$^+$ Treg led almost to a doubling in their frequencies. Frequencies of autoantigen-specific CD4$^+$ T cells were found to be lower in immunized haploidentical non-autoimmune strains and increased frequencies were detectable in unmanipulated NZB/W F1 mice with active disease. *In vitro* re-addition of CD25$^+$ Treg after Treg depletion restored suppression of autoantigen-specific CD4$^+$ T cell activation.

Discussion: These results suggest that the activation and expansion of autoantigen-specific CD4$^+$ T cells are partly controlled by Treg in murine lupus. Depletion of Treg therefore can be a useful approach to increase the detectability of autoantigen-specific CD4$^+$ T cells allowing their detailed characterization including lineage determination and epitope mapping and their sufficient *ex vivo* isolation for cell culture.

KEYWORDS

autoantigen-specific T cells, immune regulation, regulatory T cells, lupus, autoimmunity

Introduction

The increase in numbers and frequencies of activated and memory-differentiated $CD4^+$ T cells in lymphatic organs and inflamed tissues in systemic lupus erythematosus (SLE) suggests that autoreactive $CD4^+$ T cells recognizing a distinct panel of autoantigens essentially contribute to immune pathogenesis (1). Among other autoantigens, the C-terminus of the SmD1 protein, the SmD1(83-119) peptide (SmD1p), is considered a key autoantigen in both murine and human SLE (2). T and B cell-driven immune responses against this particular self-peptide can be detected in the majority of SLE patients as well as in mouse models for lupus (3, 4). Despite the well-accepted importance of autoantigen-specific $CD4^+$ T cells in lupus, little is known about their characteristics and how their activation and expansion are controlled.

Different techniques have been used to identify autoantigen-specific $CD4^+$ T cells to date; however, the reliable detection and isolation of autoantigen-specific $CD4^+$ T cells are strongly limited due to the very low prevalence of these cells, which is considered to be lower than 1 per 10^5 T cells (5). These low frequencies often do not allow the isolation and characterization of autoantigen-specific $CD4^+$ T cells. The use haplotype dependent MHCII-peptide multimers to detect antigen-specific CD4+ T cells is still substantially restricted due to the common unavailability of suitable constructs. Other approaches, such as ELISPOT assays for cytokine secretion or flow cytometric intracellular cytokine staining used to detect antigen-specific $CD4^+$ T cells do not allow for the isolation of living cells. Recently, CD154, also known as CD40L, has been demonstrated to be a reliable marker for the identification of antigen-specific $CD4^+$ T cells (6). The transient expression of CD154 on the surface of $CD4^+$ T cells upon antigenic stimulation can be used to assess living antigen-specific $CD4^+$ T cells in both human and animal models (6, 7). For the successful isolation of antigen-specific $CD4^+$ T cells, investigators have often used foreign antigens. However, the detection of autoantigen-specific $CD4^+$ T cells in systemic autoimmune diseases is even more challenging since endogenous mechanisms of self-tolerance, such as the suppression of autoimmune responses by $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg), may inhibit their activation in the ubiquitous presence of autoantigens. Meanwhile, there is a general consensus that Treg are indispensable for controlling autoimmunity by keeping autoreactive conventional T cells (Tcon) and other harmful immune cells in check (8). Recent studies indicate that Treg exhibit a higher avidity for autoantigens compared to conventional $CD4^+$ T cells (Tcon) (9). Furthermore, it is generally assumed that Treg can prevent the activation of naïve $CD4^+$ T cells and their differentiation into effector/memory $CD4^+$ T cells. Other studies also suggested a suppressive effect of Treg on already differentiated effector/memory $CD4^+$ T cells (10).

SLE is characterized by progressive loss of Treg-mediated control over effector/memory Tcon due to an acquired deficiency of the Treg growth and survival factor IL-2 resulting in an imbalance between Treg and Tcon that advances during the disease course (11–14). Hence, the adoptive transfer of $CD4^+FoxP3^+CD25^+$ Treg into NZB/W F1 mice with established disease or expansion of endogenous Treg by IL-2 delayed disease progression and increased the survival time (11, 15, 16). By contrast, *in vivo* depletion of $CD25^+$ Treg using a depleting anti-CD25 antibody accelerated the development of murine lupus (11). However, in SLE, as a disease with autoreactivity toward several autoantigens, the close interaction between Treg and autoantigen-specific $CD4^+$ effector T cells remains to be elucidated. As shown before, immunization of lupus-prone mice against the SmD1p led to a strong disease acceleration (3, 17). Taking this into account, continuous activation and expansion of autoantigen-specific $CD4^+$ Tcon may reflect a failure of the corresponding Treg to control their activation adequately. However, the tight control of autoantigen-specific effector $CD4^+$ Tcon by Treg cells could also account for the difficulties in reliably identifying these rare cells. Concerning this matter, we could show in a previous work that *in vitro* depletion of $CD25^+$ Treg prior to antigen stimulation led to the unmasking of autoreactive $CD4^+$ T cell responses against SmD1p in patients with SLE (18). To provide more in-depth evidence for the inhibitory role of Treg in an autoantigen-specific context in lupus, we investigated here whether the *in vitro* and *in vivo* removal of Treg could facilitate the detection and characterization of autoantigen-specific effector/memory $CD4^+$ T cells in the NZB/W F1 mouse model for lupus.

Materials and methods

Mice

Lupus-prone female New Zealand Black × New Zealand White F1 (NZB/W F1) and non-lupus-prone female Balb/c x New Zealand White F1 (CW F1) mice were obtained from the Federal Institute for Risk Assessment, Berlin, Germany, and the Research Facility for Experimental Medicine of the Charité – University Medicine, Berlin, Germany. The mice were kept in a specific pathogen-free (SPF) environment at the German Rheumatism Research Centre (DRFZ), Berlin, Germany. All experiments were performed according to institutional and federal guidelines (State Office for Health and Social Affairs, Berlin, Germany). NZB/W F1 mice of different disease stages were used: young, clinically healthy mice (8-12 weeks of age, in average 10.4 weeks, no proteinuria); mice at disease onset (5-7 months of age, in average 6.4 months, proteinuria = 30-100 mg/dL); mice with established disease (7-9 months of age, in average 8.8 months, proteinuria = 300-2000 mg/dL). Each mouse was analyzed individually.

Antigens

The SmD1(83-119) peptide (=SmD1p) (VEPKVKSKKREAVA GRGRGRGRGRGRGRGRGGPRR) and a control peptide (= control) with amino acids identical to the SmD1(83–119) peptide, however, with a random sequence of the same amino acids (CREKGRVGRGRPAVGRRGVGRPGRRGSRARGEGKGRK), were synthesized according to a standard procedure. For $CD4^+$ T cell epitope mapping 7 overlapping 15-mer peptides derived from SmD1(83-119) [epitope 1 = SmD1(83–97), -epitope 2 = SmD1(85–

99), -epitope 3 = SmD1(87–101), -epitope 4 = SmD1(90–104), -epitope 5 = SmD1(93–107), -epitope 6 = SmD1(97–111), and -epitope 7 = SmD1(105–119)] were synthesized by JPT Peptide Technologies GmbH, Berlin/Germany. An 11-mer peptide derived from hen egg lysozyme (HEL (106-116) = NAWVAWRNRCK) was used as control for the 15-mer SmD1 peptides. All peptides were solved in PBS, sterile filtered by 0,2µm filters, and tested negative for the presence of endotoxins.

Generation of SmD1p-specific CD4$^+$ T cells by immunization of NZB/W F1 mice and CW F1 mice

For the *in vivo* generation of SmD1p-specific CD4$^+$ T cells, 8-10 weeks old female NZB/W F1 mice or 8-10 weeks old female CW F1 mice were immunized intravenously in the tail vein with 25µg of SmD1(83-119) together with 10µg of lipopolysaccharide (LPS, from E. coli 026:B6*C – Sigma-Aldrich, Taufenkirchen/Germany) as adjuvant diluted in 100µl PBS. After 8 days, a booster immunization was performed subcutaneously in the tail base with 25µg SmD1(83-119) diluted in PBS and emulsified with incomplete Freund's adjuvant (IFA) (1:1) in a total volume of 50 µl. Another 7 days later, mice were sacrificed and spleen cells were harvested.

In vivo depletion of CD25$^+$ Treg

For the *in vivo* depletion of CD25$^+$ Treg, 8-10 weeks old female NZB/W F1 or CW F1 mice were injected i.v. with 200µg of an anti-CD25-antibody (rat IgG1, clone PC61.5, DFRZ, Berlin/Germany) one week before the initial immunization. The success of depletion was confirmed by flow cytometry in peripheral blood cells using a non-cross-reactive anti-CD25 antibody (clone 7D4, DRFZ, Berlin/Germany).

Isolation of splenocytes

Spleens from NZB/W F1 or CW F1 mice were mashed individually through a 70µm cell strainer (BD Biosciences, Heidelberg/Germany), and the cell suspension was washed once with PBS/0,2% BSA. Erythrocytes were eliminated by lysis using a solution containing 0,01M KHCO3, 0,155M NH4Cl, and 0.1mM EDTA at pH 7.5 followed by washing with PBS/0,2% BSA/2mM EDTA.

In vitro depletion of CD25$^+$ cells

Erythrocyte-depleted splenocytes were incubated with monoclonal PE-conjugated anti-CD25 antibody (7D4) followed by anti-PE MicroBeads (both Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CD25$^+$ cells were depleted using the AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD25$^-$ fraction showed a purity of over 99% as analyzed by flow cytometry.

Short-term *in vitro* re-stimulation with antigens

For the detection of antigen-specific CD4$^+$ T cells and CD4$^+$ T cell epitope mapping, splenocytes from un-depleted, *in vivo* CD25$^+$ depleted, *in vitro* CD25$^+$ depleted and both *in vivo* and *in vitro* CD25$^+$ depleted mice were used. Cells were re-stimulated *in vitro* with SmD1p, 15-mers derived from SmD1p, or with control peptides (randomized peptide, HEL) at a concentration of 20µg/ml for 6h in AIM-V medium (Invitrogen, Darmstadt/Germany) in 96-well plates at a cellular concentration of 1x10^7 cells/ml. For the comparison between surface and intracellular staining of CD154, different concentrations ranging between 5 µg/ml and 100 µg/ml of SmD1p were used for *in vitro* re-stimulation.

Staining procedures for the detection of antigen-specific CD4$^+$ T cells by flow cytometry

For intracellular staining of CD154$^+$ cells, 5µg/ml brefeldin A (Sigma-Aldrich, Hamburg/Germany) was added 2 hours after the addition of antigens to the cell culture. After additional 4 hours of stimulation, cells were fixed in 2% paraformaldehyde for 20 min at room temperature, washed three times, and kept in PBS containing 0,2% BSA and 0,01% sodium azide at 4°C until FACS analyses were performed. Fixed cells were permeabilized in PBS/0,2% BSA containing 0.5% saponin and stained intracellularly using allophycocyanin (APC)-conjugated anti-CD154 antibodies (clone MR1, Miltenyi Biotec, Bergisch Gladbach/Germany). Anti-Fc-γ-receptor antibody (clone 2.4G2, 40µg/ml, DRFZ, Berlin/Germany) was added during the staining procedure to avoid unspecific binding. For surface staining of CD154, unconjugated anti-CD40 antibody (clone 1C10, 10µg/ml, BioLegend, San Diego/USA), anti-Fc-γ-receptor antibody (clone 2.4G2, 40µg/ml, DRFZ, Berlin/Germany) and APC-conjugated anti-CD154 antibody (clone MR1, Miltenyi Biotec, Bergisch Gladbach/Germany) were added to the culture from the beginning. After intracellular staining of CD154 or after the *in vitro* re-stimulation period (6h) for the surface staining of CD154, cells were washed with PBS/0,2% BSA and stained with FITC-conjugated anti-CD4 antibody (clone RM4-5, 5 µg/ml, BD Pharmingen, Heidelberg/Germany) on ice for 15 minutes. Stained cells were analyzed with a FACSCalibur 4-color flow cytometer (BD Biosciences, Heidelberg/Germany).

Staining procedure for intracellular cytokine detection by flow cytometry

Fixed cells were permeabilized in PBS/0,2% BSA containing 0.5% saponin and stained for intracellular cytokines and surface markers. Anti-Fc-γ-receptor antibody (clone 2.4G2, 40µg/ml,

DRFZ, Berlin/Germany) was used to avoid unspecific binding. The following monoclonal antibodies were used for intracellular and surface staining: APC-conjugated anti-CD154 (clone MR1, Miltenyi Biotec, Bergisch Gladbach/Germany), FITC-conjugated anti-IFN-γ (clone XMG1.2, DRFZ, Berlin/Germany), FITC-conjugated anti-TNF-α (clone MP6-XT22, DRFZ, Berlin/Germany), PE-conjugated anti-IL-2 (clone JES6-5H4, DRFZ, Berlin/Germany), PE-conjugated anti-IL-10 (clone JES5-16E3, DRFZ, Berlin/Germany), PE-conjugated anti-IL-17 (clone TC11-18H10, DRFZ, Berlin/Germany), Streptavidin-PerCP (BD Pharmingen, Heidelberg/Germany) and biotin-conjugated anti-CD4 (clone GK1.5, DRFZ, Berlin/Germany). FoxP3-staining was performed using FITC- or PE-conjugated anti-FoxP3 (clone FJK-16s, eBioscience, San Diego/USA) according to the manufacturer's instructions. Stained cells were analyzed with a FACSCalibur 4-color flow cytometer (BD Biosciences, Heidelberg/Germany).

Statistical analysis

GraphPad Prism 5 software (Windows version, GraphPad Software Inc., La Jolla/USA) was used for statistical analyses. The two-tailed paired t-test was used to compare differences between SmD1p and control peptide-stimulated samples. Mann-Whitney test was used for the comparison of frequencies of SmD1p-specific $CD4^+$ T cells between CW F1 and NZB/W F1 mice and to compare differences in the percentages of $FoxP3^+$ and $FoxP3^-$ cells among $CD4^+CD25^+$ splenocytes at different disease stages in NZB/W F1 mice. Two-way ANOVA was used to compare differences in the frequencies of SmD1p-specific $CD4^+$ T cells between undepleted, *in vitro* depleted, *in vivo* depleted and *in vivo* and *in vitro* $CD25^+$ depleted samples and between controls. Two-way ANOVA was also used for the comparisons between surface and intracellular staining of CD154. The statistical tests used for each experiment are indicated in the respective figure legends. P values of < 0.05 were considered statistically significant.

Results

Discrimination of autoantigen-specific $CD4^+$ T cells by intracellular staining for CD154 is superior to cytokine staining

Our previous works suggested that the highest frequencies of SmD1p-specific $CD4^+$ T cells could be generated by immunization of NZB/W F1 mice against the autoantigen (3). Thus, we primarily used splenocytes from immunized mice for the detection of SmD1p-specific $CD4^+$ T cells after antigenic *in vitro* re-stimulation. Firstly, we aimed to compare the yield of detectable SmD1p-specific $CD4^+$ T cells obtained by conventional intracellular staining for the Th1 lineage cytokine IFN-γ, the most abundant pro-inflammatory $CD4^+$ T cell cytokine in murine lupus (11, 19), with intracellular staining for CD154. Intracellular staining for IFN-γ yielded a mean frequency of 0.32% (range: 0.18-0.40%) of SmD1p-specific IFN-γ^+ cells among $CD4^+$ T cells which were significantly higher than the percentages of $CD4^+$IFN-γ^+ T cells obtained by stimulation with the control peptide (mean 0.16%; p=0.02, SmD1p vs control; Figure 1A). However, the unspecific background response obtained by re-stimulation with the control peptide was relatively high representing app. 50% of IFN-γ^+ cells obtained by re-stimulation with SmD1p. In addition, the staining was diffuse with a high variability in fluorescence intensity and high standard deviations (app. 0.1%) among the individually analyzed mice. Using intracellular staining for CD154 yielded a mean frequency of 0.12% (range: 0.10-0.13%) of SmD1p-specific $CD154^+$ cells among $CD4^+$ T cells. Although the frequencies of detectable antigen-specific $CD4^+$ T cells were lower compared to the IFN-γ^+ staining, the unspecific background activation was nearly absent when using the CD154 staining approach (mean 0.02% for re-stimulation with control peptide) and standard deviations were low, providing also a higher level of statistical significance for the difference between SmD1p and control peptide stimulated samples (p=0.01, Figure 1B). In addition, the discrimination of positively stained $CD4^+$ T cells from negatively stained $CD4^+$ T cells was superior by using the CD154 staining approach in comparison to conventional cytokine staining. Because of the more reliable detection and superior background discrimination of autoantigen-specific $CD4^+$ T cells, we used the CD154 intracellular staining approach for the detection of autoantigen-specific $CD4^+$ T cells in our further experiments.

Depletion of $CD25^+$ Treg increases the frequency of detectable autoantigen-specific $CD4^+$ T cells

Treg are known for their capability to suppress T cell responses, in particular against autoantigens, in order to efficiently counteract autoimmunity (8). To investigate whether Treg are capable to suppress SmD1p-specific $CD4^+$ T cell responses we first depleted $CD25^+$ cells *in vitro* from splenocytes of immunized NZB/W F1 mice. Efficient depletion of $CD25^+$ cells, of which app. 90% expressed the Treg lineage marker FoxP3 and co-expressed CD4, among the $CD4^+FoxP3^+$ Treg population was confirmed before antigenic re-stimulation (Figure 2A, left). Upon re-stimulation with SmD1p, samples depleted of $CD25^+$ Treg showed an average increase in the frequency of detectable $CD4^+CD154^+$ T cells by 0.04% compared to un-depleted samples (mean 0.16% vs 0.12%, p=0.0044), whereas the response to the control peptide remained low (mean 0.03%, p<0.0001, SmD1p vs control peptide) comparable to this of un-depleted samples (Figures 2A, C right).

To further substantiate this putative inhibitory effect of Treg on antigen-specific T cell responses, we now depleted $CD25^+$ Treg *in vivo* by injecting depleting anti-CD25 antibodies before immunization of NZB/W F1 mice against SmD1p (Figure 2B, left). *In vivo* depletion of $CD25^+$ Treg resulted in an average increase in the frequency of detectable $CD4^+CD154^+$ T cells by 0.05% upon *in vitro* re-stimulation with SmD1p compared to un-depleted samples (mean 0.17% vs 0.12%, p=0.0017) (Figures 2B, C, right). This increase in the frequency of SmD1p-specific $CD4^+$ T cells was comparable to the increase obtained by the *in vitro*

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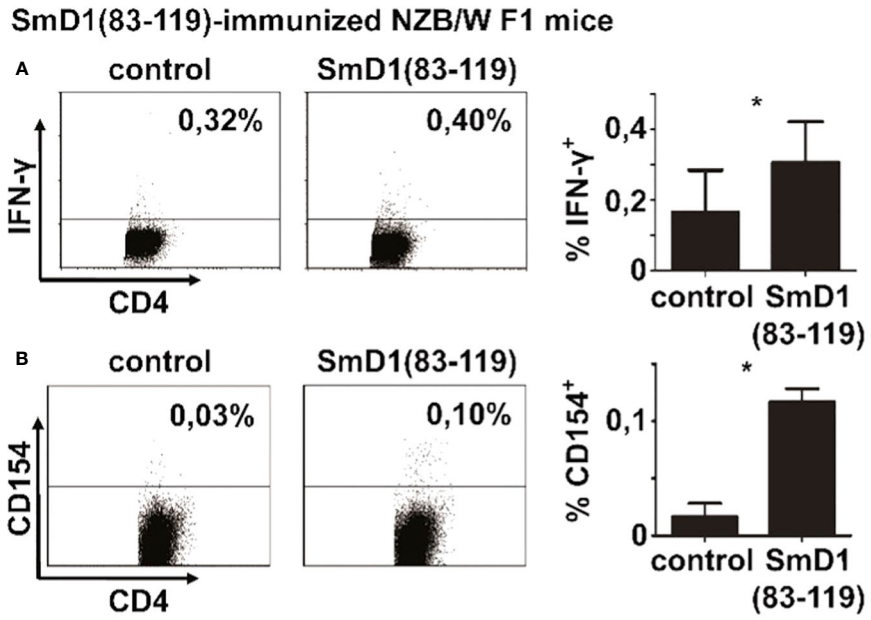

FIGURE 1
Detectability of autoantigen-specific CD4$^+$ T cell responses by different intracellular staining approaches. Splenocytes from SmD1(83-119)-immunized female NZB/W F1 mice at the age of 8-10 weeks were re-stimulated *in vitro* with SmD1(83-119) or a randomized control peptide (= control). Representative dot plots and bar diagrams show the frequencies of SmD1(83-119)-specific CD4$^+$ T cells obtained by intracellular cytokine staining for IFN-γ **(A)** and by intracellular staining for CD154 **(B)**. Data in bar diagrams are mean + SD (A, n=6 and B, n=3 per group) derived from 1 or 2 out of 2 independent experiments with 3 individual mice analyzed in each experiment. Two-tailed paired t-test was used (*p<0.05).

depletion approach (difference not significant). Similar to the *in vitro* depletion experiments, the response to the control peptide remained almost absent (mean 0.02%, p<0.0001; SmD1p vs control peptide; Figure 2C, right).

In light of these results, we asked whether the combination of both approaches, the *in vivo* and *in vitro* depletion of CD25$^+$ Treg, could have an additive effect on autoantigen-specific CD4$^+$ T cell responses. Indeed, the combined depletion approach resulted in the detection of the highest frequencies of CD154$^+$ cells among CD4$^+$ T cells with a mean frequency of 0.20% (Figure 2C), representing an increase of SmD1p-specific CD4$^+$ T cells by app. 0.08% compared to un-depleted samples (p=0.0002) and by app. 0.3% and 0.4% compared to *in vitro* (p=0.009) and *in vivo* (p=0.0293) depleted samples, respectively. Again, there was no relevant response to the control peptide (mean 0.03%, p<0.0001; SmD1p vs control peptide; Figure 2C) allowing for the reliable discrimination between positive and negative cells.

Frequencies of autoantigen-specific CD4$^+$ T cells are lower in haploidentical non-autoimmune strains and correspond to disease activity in NZB/W F1 mice

To address whether our observations are specifically associated with susceptibility for lupus, we analyzed for the presence of SmD1p-specific CD4$^+$ T cells in the genetically related mouse strain (BALB/c x NZW) F1 (CW F1) which bears the same MHC haplotypes as the NZB/W F1 strain but does not develop lupus-like disease. Immunized CW F1 mice at the same age as NZB/W F1 were subjected to a combined *in vivo* (before immunization) and *in vitro* depletion of CD25$^+$ Treg, as described above for the NZB/W F1 mice. Although, SmD1p-specific CD4$^+$ T cells according to CD154 expression were detectable above background in CW F1 mice upon *in vitro* re-stimulation (mean 0.09% vs 0.02%, p=0.002, SmD1p vs control peptide; Figure 3A) their frequency was more than 2-fold lower compared to the responses observed in Treg depleted NZB/W F1 lupus-prone mice (p=0.002; CW F1 vs NZB/W mice; Figure 3A).

To obtain insights into the relevance of SmD1p-specific CD4$^+$ T cells and the inhibitory potential of Treg on autoantigen-specific CD4$^+$ T cell responses in disease pathogenesis, we examined whether SmD1p-specific CD4$^+$ T cells can be also detected and their frequency increased by depletion of CD25$^+$ Treg during spontaneous disease development. Accordingly, splenocytes from non-immunized NZB/W F1 mice at different disease stages were *in vitro* depleted from CD25$^+$ Treg or left un-depleted before stimulation with SmD1p. In young, clinically healthy NZB/W F1 mice no relevant frequencies of SmD1p-specific CD4$^+$CD154$^+$ T cells above background levels could be observed, neither in un-depleted nor in depleted samples (Figure 3B). Although, frequencies of SmD1p-specific CD4$^+$CD154$^+$T cells were higher in un-depleted samples of NZB/W F1 mice at the onset of the disease compared to young NZB/W F1 mice, there was no significant difference compared to the control peptide re-stimulation in both un-depleted and depleted samples (Figure 3B). However, in mice with established disease there was a significantly higher frequency of SmD1p-specific CD4$^+$ T cells in un-depleted samples compared to the stimulation with the control peptide (p<0.05) and the frequency reached similar levels as observed in young

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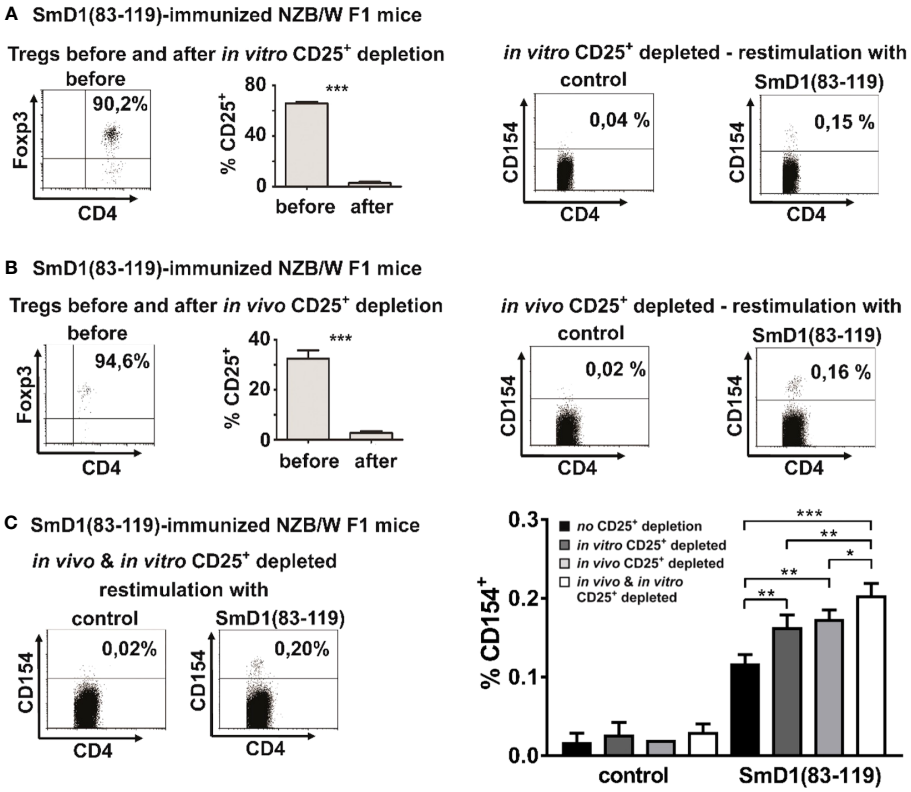

FIGURE 2
Detection of autoantigen-specific $CD4^+$ T cells using different Treg depletion strategies. **(A, B)** NZB/W F1 mice were immunized against SmD1(83-119) and splenocytes were *in vitro* depleted from $CD25^+$ cells by MACS **(A)** or NZB/W F1 mice were *in vivo* depleted from $CD25^+$ cells by injection of depleting anti-CD25 antibodies (clone PC61) before immunization **(B)**. Dot plots in left panels show the percentage of FoxP3+ cells among gated $CD4^+CD25^+$ T cells before *in vitro* **(A)** or *in vivo* **(B)** depletion of $CD25^+$ cells and bar diagrams compare the percentages of $CD25^+$ cells among $CD4^+FoxP3^+$ cells before and after depletion of $CD25^+$ cells (n=3-5 per group). Representative dot plots on the right side show the percentages of $CD154^+$ cells among gated $CD4^+$ splenocytes from *in vitro* **(A)** or *in vivo* **(B)** CD25 depleted mice upon *in vitro* re-stimulation with SmD1(83-119) or the control peptide (= control). **(C)** Representative dot plots on the left side show the percentages of $CD154^+$ cells among gated $CD4^+$ splenocytes from combined *in vitro* and *in vivo* $CD25^+$ depleted NZB/W F1 mice upon *in vitro* re-stimulation with SmD1(83-119) or the control peptide (= control). Bar diagram on right side summarizes results obtained by re-stimulation with SmD1(83-119) or the control peptide (= control) using different Treg depletion strategies compared to un-depleted samples. Data in bar diagram are mean + SD (n=3 per group) derived from 1 out of 2 independent experiments with 3 individual mice analyzed in each experiment. Female NZB/W F1 mice at the age of 8-10 weeks were used in all experiments. Two-tailed paired t-test was used for diagrams shown in Figures 2A, B, two-way ANOVA was used for bar diagram shown in Figure 2C (*p<0.05, **p<0.01, ***p<0.001).

mice after immunization with SmD1p, although a much higher level of unspecific background staining for CD154 was evident in the controls suggesting a higher state of constitutive T cell activation in mice with active disease (Figure 3B). Unexpectedly, this difference was no more detectable after *in vitro* depletion of $CD25^+$ cells as the frequency of SmD1p-specific $CD4^+CD154^+$ T cells dropped to the same level obtained by stimulation with the control peptide. Since CD25 is not exclusively expressed on Treg and is known to be expressed also on activated conventional $CD4^+$ T cells (Tcon), and frequencies of $CD25^+$ cells among $CD4^+FoxP3^-$ Tcon were shown to be increased in NZB/W F1 mice with active disease (11), the observed loss of SmD1p-specific $CD4^+$ T cells after depletion of $CD25^+$ cells in diseased mice could be explained by the depletion of the $CD4^+CD25^+$ Tcon population containing also activated SmD1p-specific $CD4^+$ T cells. Thus, we analyzed the percentages of $FoxP3^+$ and $FoxP3^-$ cells within the $CD4^+CD25^+$ T cell population, respectively, at different disease stages. In line with our assumption, the percentages of $FoxP3^+$ Treg among $CD4^+CD25^+$ T cells were significantly reduced in diseased NZB/W F1 mice compared to young (p=0.0019) and onset (p=0.04) mice, whereas the percentages of $FoxP3^-$ Tcon among $CD4^+CD25^+$ T cells were significantly higher compared to young (p=0.0019) and onset (p=0.04) mice (Figure 3C).

In vitro re-addition of $CD25^+$ Treg after Treg depletion restores suppression of autoantigen-specific $CD4^+$ T cells

To exclude that the observed increase in the frequency of autoantigen specific $CD4^+$ T cells by depletion of $CD25^+$ Treg is biased due to changes in the cellular composition induced by the removal of $CD25^+$ cells, we tested whether the *in vitro* re-addition of $CD25^+$ Treg after Treg depletion is capable to restore Treg-mediated suppression of autoantigen-specific T cell responses by decreasing the percentage of detectable SmD1p-specific $CD4^+$ T cells again. Accordingly, we used splenocytes from *in vivo* and *in vitro* $CD25^+$ depleted and immunized

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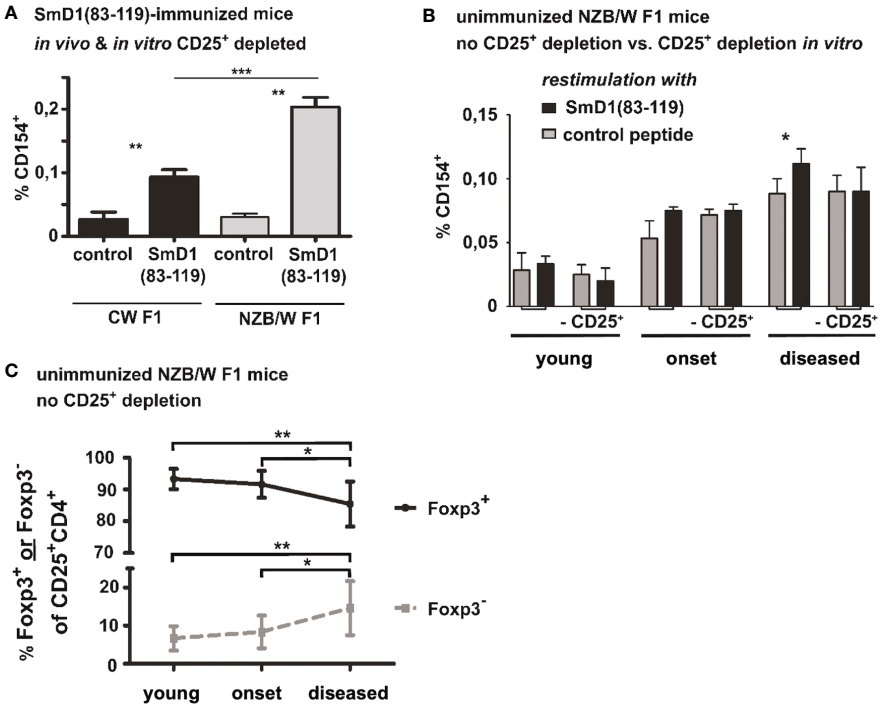

FIGURE 3
Frequencies of autoantigen-specific CD4$^+$ T cells in haploidentical non-autoimmune CW F1 mice and their association with disease activity in NZB/W F1 mice. **(A)** Comparison of SmD1(83-119)-specific CD4$^+$ T cell responses in SmD1(83-119)-immunized lupus-prone NZB/W F1 mice versus non-lupus-prone CW F1 mice after combined *in vivo* and *in vitro* depletion of CD25$^+$ Treg. Frequencies of CD154$^+$ cells among CD4$^+$ T cells upon re-stimulation of splenocytes with SmD1(83-119) or the control peptide are shown. Female mice at the age of 8-10 weeks were used for both strains. **(B)** Detection of autoantigen-specific T cell responses during spontaneous disease development in unimmunized NZB/W F1 mice. Frequencies of SmD1(83-119)-specific CD4$^+$CD154$^+$ T cells from young, healthy mice (no proteinuria, age 8-12 weeks), mice at the onset of disease (proteinuria = 30-100 mg/dL, age 5-7 months), and mice with established disease (proteinuria = 300-2000 mg/dL, age 7-9 months) are shown for un-depleted and *in vitro* CD25$^+$ depleted samples upon re-stimulation of splenocytes with SmD1(83-119) or control peptide (= control). **(C)** Percentages of FoxP3$^+$ and of FoxP3$^-$ cells among gated CD4$^+$CD25$^+$ splenocytes at different disease stages in unmanipulated NZB/W F1 mice. (n=9 mice per age group) (A, B) Shown data are mean + SD (n=3 per group) derived from 1 out of 2 independent experiments with 3 individual mice analyzed in each experiment. Two-tailed paired t-test was used to compare differences between SmD1(83-119) and control peptide stimulated samples **(A, B)**. Mann-Whitney test was used for comparison of frequencies of SmD1(93-119)-specific CD4+ T cells between CW F1 and NZB/W F1 mice **(A)** and to compare differences in the percentages of FoxP3$^+$ and FoxP3$^-$ cells among CD4$^+$CD25$^+$ splenocytes at different disease stages in NZB/W F1 mice **(C)** (*p<0.05, **p<0.01, ***p<0.001).

NZB/W F1 mice and re-added either none, 5%, or 10% of purified CD25$^+$ Treg from unmanipulated, 8-10 weeks old NZB/W F1 mice (total cell number in the cell culture remained the same in all settings) before *in vitro* re-stimulation with SmD1p. Indeed, the re-addition of 10% of purified CD25$^+$ Treg resulted in a decrease in the percentages of detectable SmD1p-specific CD4$^+$CD154$^+$ T cells from a mean of 0.15% to a mean of 0.09% (p=0.01, Figure 4), which corresponds to frequencies obtained by stimulation without prior Treg depletion. The re-addition of 5% of purified CD25$^+$ Treg, however, was not sufficient to reduce the percentages of SmD1p-specific CD4$^+$CD154$^+$ T cells.

Depletion of CD25$^+$ Treg facilitates deeper analysis of autoantigen-specific CD4$^+$ T cells

Next, we aimed to delineate the lineage differentiation by determining the cytokine profile of SmD1p-specific CD4$^+$CD154$^+$ T cells in splenocytes from immunized NZB/W F1 mice that were subjected to the combined Treg depletion approach. We found that SmD1p-specific CD4$^+$CD154$^+$ T cells produced mainly TNF-α (mean frequency 53%) and IFN-γ (mean frequency 48%), and to a much lesser extent IL-10 and IL-17 (mean frequency < 4% for both), indicating that the majority of SmD1p-specific CD4$^+$ T cells belonged to the Th1 lineage (Figure 5A).

We also performed fine epitope mapping with 7 different overlapping 15-mer peptide sequences derived from the SmD1p to determine the pre-dominant CD4$^+$ T cell epitope. We compared the frequencies of antigen-specific CD4$^+$CD154$^+$ T cells obtained from immunized NZB/W F1 mice without depletion of CD25$^+$ Treg to those from immunized NZB/W F1 mice with combined *in vivo* and *in vitro* depletion. In both experimental settings, re-stimulation with the SmD1p sequence 3 (amino acids 87-101) provided the highest frequencies of epitope-specific CD4$^+$CD154$^+$ T cells (mean 0.04%, p=0.03 for un-depleted vs control; mean 0.11%, p=0.001 for CD25$^+$ depleted vs control; Figure 5B). Notably, in the CD25$^+$ depleted samples the frequencies of epitope 3-specific CD4$^+$CD154$^+$ T cells were significantly higher than in the un-depleted samples (p=0.007; Figure 5B). Apart from the epitope sequences 2 and 4,

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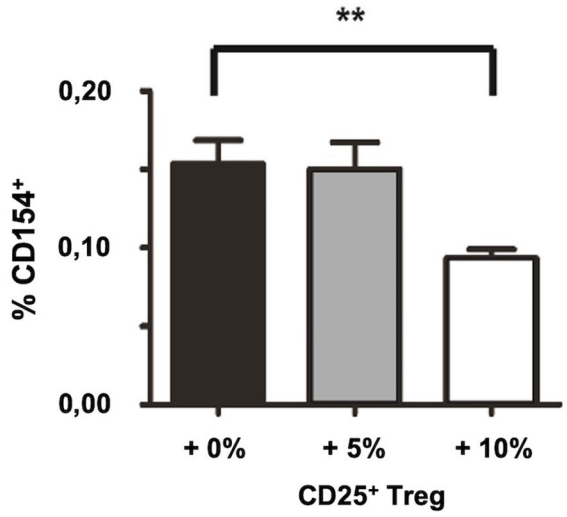

FIGURE 4
Restoration of suppression of autoantigen-specific $CD4^+$ T cell responses by *in vitro* re-addition of $CD25^+$ Treg after $CD25^+$ Treg depletion. Bar diagram summarizes the frequencies of SmD1(83-119)-specific $CD4^+CD154^+$ T cells upon re-addition of the indicated percentages of $CD25^+$ Treg (0, 5, or 10%, respectively) into the cultures before re-stimulation with SmD1(83-119). Female NZB/W F1 mice at the age pf 8-10 weeks were used for all experiments. Shown data are mean + SD (n=3 per group) derived from 1 out of 2 independent experiments with 3 individual mice analyzed in each experiment. Two-tailed paired t-test was used (**p<0.01).

which elicited a moderate increase in the frequency of $CD4^+CD154^+$ T cells in $CD25^+$ depleted samples, no relevant T cell responses to other peptide sequences were observable (Figure 5B). Thus, in NZB/W F1 mice epitope sequence 3 was identified as the dominant $CD4^+$ T cell epitope within the entire SmD1p sequence.

One advantage in the detection of (auto)antigen-specific $CD4^+$ T cells by CD154 staining and flow cytometry is the possibility to stain CD154 on the surface of living cells, in order to isolate antigen-specific $CD4^+$ T cells alive with only little manipulations of these cells. Thus, we compared the frequencies of SmD1p specific CD4+ T cells obtained by surface staining of CD154 with those obtained by the intracellular staining approach by using increasing concentrations of the SmD1p antigen. The surface staining of CD154 on living SmD1p-specific $CD4^+$ T cells in general revealed lower frequencies of these cells compared to the intracellular CD154 staining approach for all antigen concentrations tested (Figure 5C). However, significantly higher frequencies of SmD1p-specific CD4+ T cells compared to the re-stimulation with the control peptide were evident for all antigen concentrations ($p=0.01$) and highest frequencies were observed by stimulation with concentrations of 20 µg/ml and 50 µg/ml. The intracellular CD154 staining approach yielded significantly higher frequencies of $CD4^+CD154^+$ T cells than the surface CD154 staining in nearly all concentrations of SmD1p used for re-stimulation ($p<0.05$ and $p<0.01$), except for the re-stimulation experiment with the concentration of 20 µg/ml (ns, $p=0.06$). Similar to what was observed with the surface staining, there was a tendency toward a dose dependency of the SmD1p-specific CD4+ T cell responses (Figure 5C).

Discussion

The detection and characterization of autoantigen-specific $CD4^+$ T cells in autoimmune diseases is challenging due to several limitations. First, despite the abundance of autoantigens in autoimmune diseases such as SLE, the frequency of autoantigen-specific $CD4^+$ T cells generally is very low and often below the detection limit using flow cytometric approaches (20). Furthermore, the presentation of autoantigens released from dying cells in cell cultures may promote continuous activation of autoantigen-specific $CD4^+$ T cells, leading to a refractory state upon further antigenic stimulation (21). Third, $CD4^+$ T cell responses to autoantigens can be actively suppressed *in vivo* or *in vitro* by Treg, which mainly recognize autoantigens via their T cell receptors (8).

Here we describe a practical approach to increase the detectability and the yield of these rare cells by flow cytometry through the removal of CD25+ Treg, which opens new possibilities for the deeper classification and enrichment of autoantigen-specific $CD4^+$ T cells in a large variety of settings and autoimmune diseases including the identification of immunodominant peptide sequences for the development of suitable MHCII-peptide multimers. In addition, we provide further evidence that Treg are indeed involved in the suppression of specific $CD4^+$ T cell responses against autoantigens in murine lupus, as the depletion of $CD25^+$ Treg, either *in vitro* before re-stimulation with the autoantigen or *in vivo* before the immunization against the autoantigen, led to an increase in the frequencies of detectable auto-antigen-specific $CD4^+$ T cells by approximately 50%. Of note, the combination of both depletion approaches yielded the highest frequencies of detectable auto-antigen-specific $CD4^+$ T cells resulting almost in a doubling of their frequencies compared to un-depleted samples. On the other hand, this additive effect could be reversed by the re-addition of Treg to the Treg-depleted cultures, pointing to the restoration of Treg-mediated suppression of autoantigen-specific $CD4^+$ T cells and confirming their inhibitory potential. Such an inhibitory effect of $CD25^+$ Treg on autoantigen-specific $CD4^+$ T cell responses and their unmasking by *in vitro* depletion of $CD25^+$ Treg before antigen stimulation was also reported for the detection of SmD1p-specific $CD4^+$ T cells in human SLE patients thereby enabling their correlation with disease activity (18).

By using this combined approach, significantly higher frequencies of autoantigen-specific $CD4^+$ T cells could be detected in lupus-prone NZB/W F1 mice compared to haploidentical CW F1 mice that are not susceptible to lupus-like disease, suggesting a possible relationship between the generation of SmD1p-specific $CD4^+$ T cells and the genetic pre-disposition for lupus. In consideration of our previous work showing a progressively impaired Treg-mediated control over $CD4^+$ Tcon activation in NZB/W F1 mice (11), it appears plausible that even in young, clinically healthy lupus-prone mice Treg control autoantigen-specific $CD4^+$ T cells to a lesser degree than in a non-lupus-prone mouse strain, which facilitates their escape and clonal expansion. In line with this, we found that frequencies of autoantigen-specific $CD4^+$ T cells increased during spontaneous disease progression in unimmunized NZB/W F1 mice, in which the highest frequencies

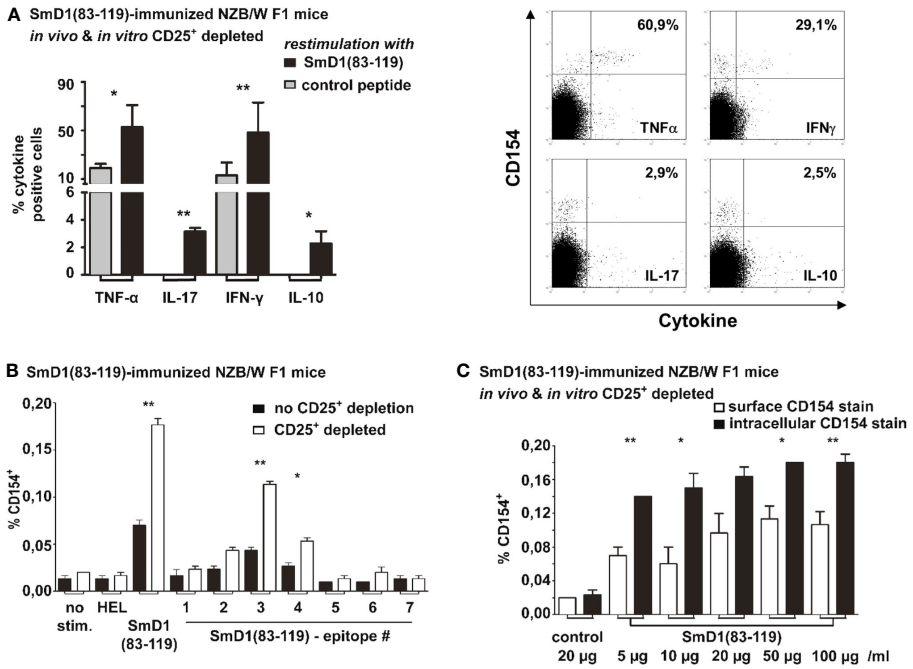

FIGURE 5
Cytokine profiling and epitope mapping of autoantigen-specific CD4$^+$ T cells and comparison of surface and intracellular staining of CD154. Combined *in vivo* and *in vitro* CD25+ Treg depletion was performed in SmD1(83-119) immunized 8-10 weeks old NZB/W F1 mice prior to re-stimulation with SmD1(83-119) or control peptide. **(A)** Percentages and representative dot plots of TNF-α, IL-17, IFN-γ and IL-10 expressing cells among SmD1(83-119)-specific CD4$^+$CD154$^+$ T cells (n=3-6 per group). **(B)** Frequencies of antigen-specific CD4$^+$CD154$^+$ T cells obtained by re-stimulation of splenocytes with HEL (11-mer peptide), SmD1(83-119), or different overlapping 15-mer peptide sequences derived from SmD1(83-119) (epitopes 1-7). Shown data are mean + SD (n=3 per group) derived from 1 out of 2 independent experiments with 3 individual mice analyzed in each experiment. **(A, B)** Two-tailed paired t-test was used (*p<0.05, **p<0.01). **(C)** Frequencies of CD154$^+$ cells among CD4$^+$ T cells are shown for the surface staining of CD154 (on living cells) or intracellular staining of CD154 (in fixed cells) using different concentrations of SmD1(83-119) or a control peptide (= control) for *in vitro* re-stimulation. Shown data are mean +SD (n=3 per group) from one experiment with 3 individual mice. Two-way ANOVA was used (*p<0.05, **p<0.01).

were detectable in mice with established disease, underlying their relevance in disease pathogenesis. This increase occurs in parallel to a progressive impairment of Treg homeostasis due to an acquired deficiency of the Treg growth and survival factor IL-2 promoting an imbalance between Treg and Tcon (11, 16), which also reflects the concomitant and progressive loss of Treg-mediated suppression of Tcon activation.

A practical advantage of using this combined depletion approach together with staining for CD154 is that autoantigen-specific CD4$^+$ T cells can be discriminated in a distinct population with higher yields and with low unspecific background staining for further characterization, such as analysis of the cytokine expression profile of autoantigen-specific CD4$^+$ T cells. Here we found that IFN-γ and TNF-α were the most abundantly expressed cytokines within the SmD1p-specific CD4$^+$ T cell population. The preferential Th1 lineage commitment of SmD1p specific CD4$^+$ T cells was somehow expected because of the use of adjuvants during the immunization procedure, which are known to promote Th1 differentiation, such as LPS. Still, these data show that our approach is principally feasible for cytokine profiling and lineage determination of auto-antigen specific T cells, which can be useful for future studies. We also used this approach to identify the predominant CD4$^+$ T cell epitope consisting of 15 amino acids derived from the complete SmD1p sequence, confirming previous results with other methods from our group (3). However, as shown here and before, stimulation with the full-length peptide still yielded the highest frequencies of autoantigen-specific CD4$^+$ T cells.

There are some limitations of our study. First, to identify relevant and pure frequencies of autoantigen-specific CD4$^+$ T cells, immunization with the autoantigen was required, which clearly outperformed the yield obtained from unimmunized mice. However, as immunization with adjuvants strongly influences the cytokine expression pattern and lineage commitment of autoantigen-specific CD4$^+$ T cells, the Th1 dominance observed in immunized mice might not necessarily represent the lineage of SmD1p-specific CD4$^+$ T cells that are generated spontaneously during disease progression, although a dominant Th1 lineage commitment was also described in previous studies in both human and murine lupus (5, 11, 16, 19, 22). Second, we targeted CD25 expressing cells by using anti-CD25 antibodies for the depletion of Treg in this study which is neither a specific marker for Treg nor sufficient for depleting the entire Treg population, as CD25 is not expressed on a large proportion of CD4$^+$FoxP3$^+$ Treg, in particular in NZB/W F1 mice in which only app. 30-60% of the CD4$^+$FoxP3$^+$ Treg population expresses CD25 (11). Therefore, the effects of a complete depletion of the entire CD4$^+$FoxP3$^+$ Treg population, e.g. by using a transgenic mouse model, on the detection of autoantigen-specific T cell responses remain to be addressed. On the other hand, CD25 is also

transiently expressed on recently activated $CD4^+FoxP3^-$ Tcon including autoantigen-specific T cells, which are likewise depleted using anti-CD25-antibodies. Indeed, in unimmunized NZB/W F1 mice with established disease, depletion of $CD25^+$ cells could not augment the detectability of autoantigen-specific $CD4^+$ T cells. This discrepancy might be best explained by the concomitant removal of activated CD25-expressing $CD4^+$ Tcon, containing also SmD1p-specific $CD4^+$ T cells, which are known to be increased in established lupus (11). Thus, Treg removal by using depleting anti-CD25 antibodies appears to have its limitations in particular when applied in disease stages with chronic hyperactivity of $CD4^+$ Tcon. Third, another possible critical point is that depletion of $CD25^+$ cells changes the composition and proportions of the analyzed cell populations and hence the observed increased frequencies of autoantigen-specific $CD4^+CD154^+$ T cells could in part be related to the proportional decrease in the Treg population. Nevertheless, the frequency of depleted $FoxP3^+CD25^+$ Treg is relatively low in NZB/W F1 mice representing app. 5% among total $CD4^+$ T cells (11) which does not reasonably explain the increase in autoantigen-specific $CD4^+$ T cells from app. 0.1% in un-depleted mice to 0.15 and 0.2% (50 to 100% increase) upon Treg depletion. In addition, the finding that the re-addition of $CD25^+$ Treg to Treg-depleted samples could reverse the increase in detectable auto-antigen-specific $CD4^+$ T cells, argues against an unspecific proportional effect and rather implies that SmD1p-specific $CD4^+$ T cell responses are actively suppressed by Treg. Still, quite high numbers of re-added $CD25^+$ Treg (10% of cells in splenocyte culture) were required to elicit this effect, which is likely to result in higher frequencies of $CD4^+FoxP3^+CD25^+$ among cultured splenocytes than in un-depleted mice. We suggest that such higher numbers of $CD25^+$ Treg are required because the re-added $CD25^+$ Treg were obtained from non-immunized mice, which might harbor lower frequencies of SmD1p-specific Treg than immunized mice. Another explanation could be that some of the re-added Treg could have changed their functional state during the sorting procedure.

Taken together, this novel combination of methods used for increasing the detectability of autoantigen-specific $CD4^+$ T cells provides a feasible approach to studying autoantigen-specific $CD4^+$ T cell responses in more depth in both mice and humans allowing their detailed characterization and sufficient *ex vivo* isolation. These results further support the concept that the activation and clonal expansion of autoantigen-specific $CD4^+$ T cells are, at least in part, under the control of $CD4^+FoxP3^+$ Treg in lupus.

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 approved by State Office for Health and Social Affairs, Berlin, Germany (LaGeSo). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SR: Data curation, Formal Analysis, Investigation, Validation, Writing – original draft. RU: Formal Analysis, Investigation, Validation, Writing – original draft. RA: Formal Analysis, Validation, Writing – original draft, Writing – review & editing. JO: Formal Analysis, Writing – original draft, Writing – review & editing. PE: Conceptualization, Data curation, Formal Analysis, Validation, Writing – original draft, Writing – review & editing. GR: Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing. JH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by funds from the German Research Foundation (DFG) within the Collaborative Research Centre 650 (SFB650; project number 10) and research grants from the Charité – University Medicine Berlin. The work and their authors were also supported by the Excellence Cluster 'Precision Medicine in Chronic Inflammation' (EXC 2167) of the Universities Kiel and Lübeck and by the Research Training Group (RTG) 2633, of the University of Lübeck both funded by the German Research Foundation (DFG).

Acknowledgments

We thank the members of the DRFZ T Cell Club and the SFB650 Tolerance Club for their help and fruitful discussions.

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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 29 November 2023
DOI 10.3389/fimmu.2023.1242860

OPEN ACCESS

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RECEIVED 19 June 2023
ACCEPTED 02 November 2023
PUBLISHED 29 November 2023

CITATION
Victor JR and Nahm DH (2023) Mechanism underlying polyvalent IgG-induced regulatory T cell activation and its clinical application: Anti-idiotypic regulatory T cell theory for immune tolerance.
Front. Immunol. 14:1242860.
doi: 10.3389/fimmu.2023.1242860

Mechanism underlying polyvalent IgG-induced regulatory T cell activation and its clinical application: Anti-idiotypic regulatory T cell theory for immune tolerance

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The regulatory T (Treg) cells constitute a functionally defined subpopulation of T cells that modulate the immune system and maintain immune tolerance through suppression of the development of autoimmune responses to self-antigens and allergic reactions to external antigens. Reduction in the number or function of Treg cells has been suggested as a key immune abnormality underlying the development of autoimmune and allergic diseases. *In vitro* studies have demonstrated that purified polyvalent immunoglobulin G (IgG) from multiple healthy blood donors can exert immunomodulatory effects on Treg cells. Incubation of polyvalent human IgG with purified $CD4^+CD25^{high}$ T cells increased the intracellular expression of interleukin (IL)-10. Intravenous administration of polyvalent human IgG induced significant expansions of $CD4^+$ $Foxp3^+$ Treg cells and clinical improvements in patients with autoimmune diseases. In human clinical trials, intramuscular administration of autologous total IgG significantly increased the percentage of IL-10-producing $CD4^+$ Treg cells in the peripheral blood of healthy subjects and provided significant clinical improvements in patients with atopic dermatitis. These results suggest a clinical usefulness of polyvalent IgG-induced activation of Treg cells in human subjects. This review proposes a new hypothesis for immune tolerance mechanism by integrating the pre-existing "idiotypic network theory" and "Treg cell theory" into an "anti-idiotypic Treg cell theory." Based on this hypothesis, an "active anti-idiotypic therapy" for allergic and autoimmune diseases using autologous polyvalent IgG (as immunizing antigens) is suggested as follows: (1) Intramuscular or subcutaneous administration of autologous polyvalent IgG produces numerous immunogenic peptides derived from idiotypes of autologous IgG through processing of dendritic cells, and these peptides activate anti-idiotypic Treg cells in the same subject. (2) Activated anti-idiotypic Treg cells secrete IL-10 and suppress Th2 cell response to allergens and autoimmune T cell response to self-antigens. (3) These events can induce a long-term clinical improvements in

patients with allergic and autoimmune diseases. Further studies are needed to evaluate the detailed molecular mechanism underlying polyvalent IgG-induced Treg cell activation and the clinical usefulness of this immunomodulatory therapy for autoimmune and allergic diseases.

KEYWORDS

immunoglobulins, T-lymphocytes, immunomodulation, regulatory T cell, atopic dermatitis, immune tolerance, allergic disease, autoimmune disease

1 Introduction

1.1 Immune tolerance and regulatory T cells

Immune tolerance is a state in which the immune system is unresponsive to foreign and self antigens that would otherwise result in a response (1). Immune tolerance is essential for maintaining immune homeostasis in healthy human subjects; defects in immune tolerance cause autoimmune and allergic diseases (1, 2).

Regulatory T (Treg) cells constitute a functionally defined subpopulation of T cells that modulate the immune system and have been suggested to play a central role in the maintenance of immune tolerance to self and foreign antigens through suppression of uncontrolled exaggerated autoimmune responses and allergic reactions that can be harmful to the host, thereby preventing the development of autoimmune and allergic diseases (1).

Treg cells are classified into natural Treg cells (nTreg cells) and induced Treg cells (iTreg cells) (3). nTreg cells arise from immature T cells in the thymus and express forkhead box P3 (Foxp3), CD4, and CD25 markers (4–6) and mediate peripheral immune tolerance through contact-dependent suppressor activity on other lymphocyte clones before they develop into full effector cells (7). iTreg cells develop after T cell maturation, do not express Foxp3, and can suppress other lymphocytes in peripheral tissues and lymph nodes but not through a contact-dependent mechanism; additionally, iTreg cells produce immunosuppressive cytokines (8). iTreg induction can be mediated through the exposure of naive $CD4^+$ T cells to transforming growth factor-β (TGF-β), retinoic acid, and antigen presentation in peripheral tissues (3). In peripheral immune tolerance, repeated antigen exposure can also lead to the induction of iTregs (8). Among iTreg cells, the interleukin (IL)-10-producing $CD4^+$ Treg cells (type 1 regulatory T cell: Tr1 cell) have been shown to play a key role in antigen-specific immune tolerance and can be induced through repeated administration of a specific antigen in humans (9–12). $Foxp3^+$ Treg cells are primarily generated in the thymus (tTreg), but they can also be generated extrathymically at peripheral sites (pTreg) (13). Treg cells induce cytokine-dependent immune suppression through the secretion of IL-10 which suppresses Type 1 (Th1) and Type 2 T helper (Th2) cells (1, 2). Based on animal studies, decreased number and/or defective function (deficiency or dysfunction) of Treg cell have been suggested as a critical immune abnormality responsible for the development of autoimmune and allergic diseases (1, 2).

1.2 Polyvalent immunoglobulin G for immune regulation

Intravenous immunoglobulin G (IVIg), that is polyvalent IgG purified from the plasma pool of multiple healthy human blood donors, has been used to treat patients with primary immunodeficiency diseases associated with reduced immunoglobulin production (14, 15). Due to its immunomodulatory effects, IVIg has also been used to treat various autoimmune and allergic diseases (16, 17). Current evidence suggests that polyvalent IgG modulates the function of various immune cells, including dendritic cells (DCs), neutrophils, monocytes, macrophages, T cells, and B cells (18). Under *in vitro* experimental conditions, incubation of polyvalent human IgG with purified $CD4^+CD25^{high}$ T cells increased the expression of intracellular IL-10 in these cells (19). Activation of Treg cells seems to be the central mechanism responsible for the immunomodulatory and anti-inflammatory effects of polyvalent IgG (20–22).

The induction or activation of $CD4^+$ Treg cells by IgG may involve diverse mechanisms considering the natural structure of these molecules. Some studies demonstrated that fragment crystallizable (Fc) regions of IgG obtained from IVIg formulations may interact with Fcγ receptors and thereby mediate part of the IVIg immunoregulatory effect (23, 24), while other report indicated that Fc fragments are not involved in this effect (25). Studies using recombinant IgG Fc fragments in murine models demonstrated a reduction in collagen-induced arthritis, idiopathic thrombocytopenic purpura, and myasthenia gravis (26–28), along with some beneficial effects in murine inflammatory neuropathy (29). The immunomodulatory effect of recombinant Fc fragments observed in the mouse model of collagen-induced arthritis could not be reproduced in macaques (28). In humans, the *in vivo* use of IVIg Fc fragments suggested a similar immunomodulatory effect of full IVIg in children with thrombocytopenic purpura (30); however, this effect could not be reproduced in *ex vivo* assays using human whole blood (31), and no other study could clarify the role of Fc fragments in human diseases (32).

The IgG fragments that can react with the antigen-binding sites (idiotypes) of pathogenic antibodies (IgG autoantibodies or IgE antibodies) in IVIg formulations have been suggested to mediate

several aspects of immunomodulation through not only neutralization of pathogenic antibodies but also through other mechanisms, such as inhibition of complement activation and suppression of cytokine, in autoimmune and allergic diseases (33–35).

1.3 Idiotypic network theory

The "idiotypic network theory" that was initially proposed as a mechanism of immune tolerance by Jerne in 1974 suggests that the idiotypes of autologous immunoglobulins are immunogenic enough to induce immune responses (anti-idiotypic immune response) in the same host (36, 37). In this theory, the induction of an autoimmune anti-idiotypic response to the original idiotype plays a physiological role in the development of the immune tolerance state of the host. In this theory, immune tolerance is not a passive state of the immune system not responding to self and foreign antigens, but an active state of the immune system maintained through continuous low-grade physiological autoimmune responses to idiotypes of circulating autologous immunoglobulins in mammalian hosts preventing the development of exaggerated immune responses to self or foreign antigens (bacteria, virus, allergens) harmful to the host (36, 38, 39). Immune tolerance is a state of well-controlled responsiveness of the immune system to specific substances or tissues that would otherwise have the capacity to destroy these substances or tissues (36, 38, 39). Immune tolerance is induced via exposure to specific antigens through T cell receptors (TCRs) (1, 2). In the "idiotypic network theory", idiotypes (variable regions with antigen specific binding capacity) of polyvalent autologous immunoglobulins and TCRs act as numerous endogenous antigens that can stimulate the immune system. These antigenic stimulations maintain numerous T cells and B cells with binding specificities to numerous antigens (both autoantigens and external antigens) and ultimately maintain an immune tolerance state (immune homeostasis).

This review focuses on mechanisms underlying the immunomodulation mediated by idiotypes of polyvalent IgG (including hypervariable regions of IgG) and not those mediated by the constant region of polyvalent IgG.

1.4 Anti-idiotypic regulatory T cell theory for immune tolerance

Unfortunately, knowledge regarding the scientific link between the pre-existing "Treg cell theory" and the "idiotypic network theory" for immune tolerance mechanisms is lacking. The mechanism underlying the cause-effect relationship between Treg cell activation and anti-idiotypic immune response has not been explained as yet. The disadvantages and weaknesses in the "idiotypic network theory" include the lack of experimental evidence regarding the actual mechanism in animals or humans through which immune tolerance is maintained and the absence of a human clinical trial demonstrating its clinical usefulness in treating autoimmune and allergic diseases. Rationale supporting the existence of "anti-idiotypic T cells" can be obtained from the "idiotypic network theory" itself. The "idiotypic network theory" suggests that the idiotypes of autologous immunoglobulins are immunogenic enough to induce immune responses (anti-idiotypic B cell and T cell immune response) in the same host (36, 37). Therefore, the pre-existence of anti-idiotypic T cells specifically recognizing peptides derived from idiotypes of autologous IgG is essential to maintain an immune tolerance state according to the "idiotypic network theory". Thus, we propose a new hypothesis regarding the immune tolerance mechanism by integrating the "idiotypic network theory" and "Treg cell theory" into an "anti-idiotypic Treg cell theory" in this review.

T cells need antigenic stimulations for activation and survival (1, 3). What are the antigens stimulating Treg cells? This review proposes an answer that major antigens stimulating Treg cells include idiotypes (or its peptides processed by antigen-presenting cells, APCs) of autologous immunoglobulin.

2 Four hypothetical models for the mechanism underlying polyvalent IgG-induced activation of Treg cells

This review proposes four hypothetical models for the mechanism underlying polyvalent IgG-induced activation of Treg cells on the basis of previous studies and our speculations (Figure 1).

[Model 1] Antigen presentation to naive T cells (Th0 cells): presentation of peptides from polyvalent IgG by APCs and differentiation of naive T cells into Treg cells (Figure 1A).

[Model 2] Antigen presentation to resting Treg cells: presentation of peptides from idiotypes of polyvalent IgG by APCs and activation of pre-existing resting anti-idiotypic Treg cells (Figure 1B).

[Model 3] Direct binding to antigens on T cells: activation of resting Treg cells by direct binding of polyvalent IgG to TCRs or unidentified antigens (Figure 1C).

[Model 4] Direct binding to antigens on APCs: direct binding of polyvalent IgG to conserved receptors or other unidentified antigens on APCs and induction of the tolerogenic phenotype of APCs facilitating differentiation of naive T cells into Treg cells (Figure 1D).

However, these four hypothetical models should be validated through future experiments. This review describes the limitations (unanswered questions) of these hypotheses to promote further studies on this topic by colleagues (Table 1).

2.1 Hypothetical model 1 and 2: activation of Treg cells by peptides from polyvalent IgG processed by APCs (main hypotheses of authors)

APCs, including DCs, take up antigens in peripheral tissues, process them into peptides through proteolysis, and load these peptides on major histocompatibility complex (MHC) class I and II molecules (40). This review suggests that APCs, including DCs, can

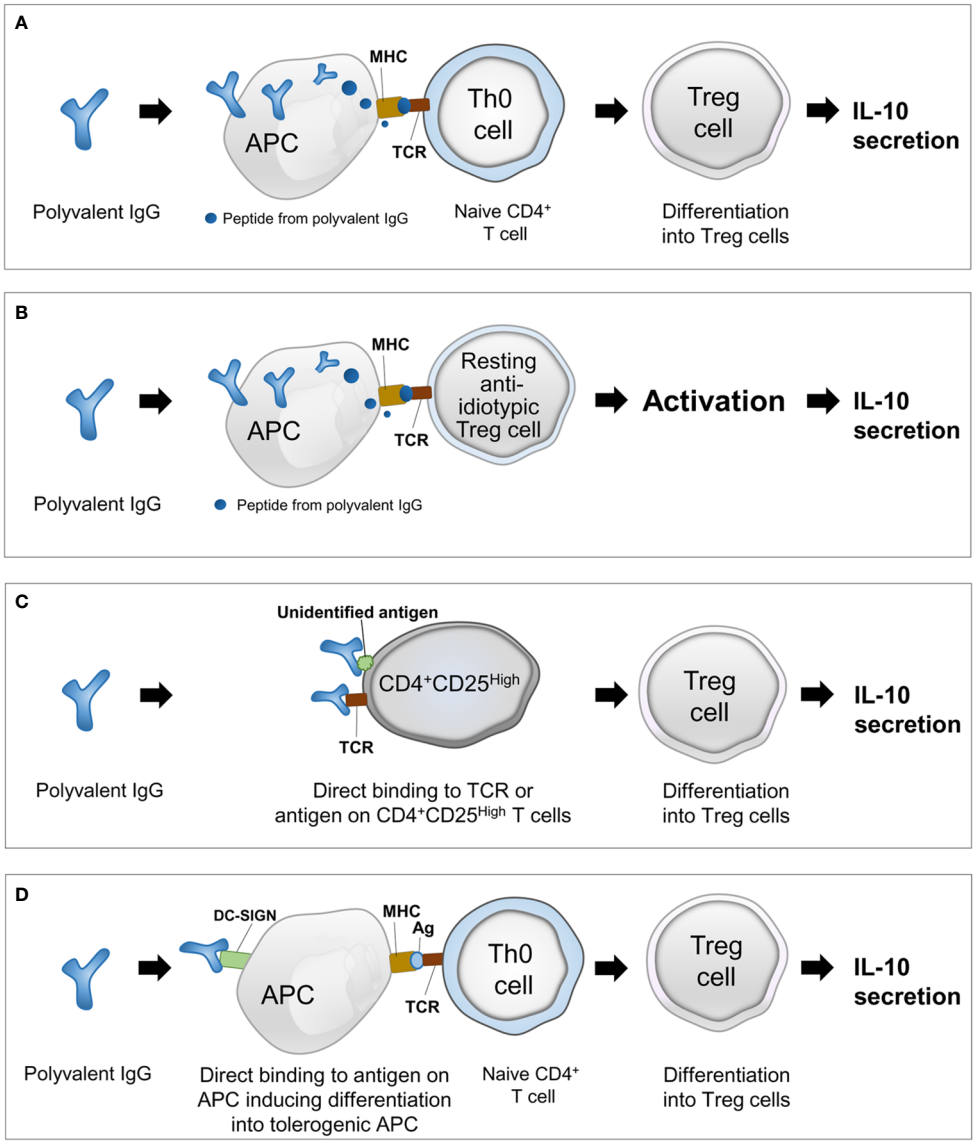

FIGURE 1
Four hypothetical models explaining the mechanism underlying polyvalent IgG-induced activation of regulatory T cells. **(A)** Presentation of peptides from polyvalent IgG by APCs and differentiation of naive T cells into Treg cells; **(B)** presentation of peptide idiotypes of polyvalent IgG by APCs and activation of pre-existing resting anti-idiotypic Treg cells; **(C)** activation of resting Treg cells by direct binding of polyvalent IgG to TCRs or unidentified antigens; and **(D)** direct binding of polyvalent IgG to conserved receptors or other unidentified antigens on APCs and induction of the tolerogenic phenotype of APCs facilitating differentiation of naive T cells into Treg cells. IgG, immunoglobulin G; Treg, regulatory T cell; IL-10, interleukin-10; TCR, T-cell receptor; APC, antigen-presenting cell; MHC, major histocompatibility complex; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin.

phagocytose polyvalent IgGs and present the peptides to naive T cells, after which the naive T cells differentiate into Treg cells [Model 1], or polyvalent IgG derived peptides activate resting anti-idiotypic Treg cells to produce IL-10 [Model 2] (Figures 1A, B). The following are past studies supporting these two hypothetical models.

In an abortion-prone mouse model, peptides produced by APCs from processing of the constant region of polyvalent IgG with a high affinity to MHC class II molecules (that can activate Treg cells and are termed as Tregitopes) can reduce the fetal death rate and increase IL-10 producing $CD4^+CD25^+Foxp3^+$ Treg cells (41). In a mouse model of ovalbumin (OVA)-induced asthma, intraperitoneal administration of IVIg from healthy human blood donors could induce antigen-specific peripherally generated $Foxp3^+$ Treg cells even after selective depletion of pre-existing $CD4^+CD25^+Foxp3^+$ Treg cells and inhibit Th2 responses and airway inflammation (42). Subsequently, the same research group demonstrated that the inhibitory effect of Tregitopes could promote tolerance by activating Treg activity in a mouse model of OVA-induced asthma (43). These results suggested that Tregitopes could be active components mediating IVIg-induced anti-inflammatory effect (Model 1, Figure 1A).

TABLE 1 Six general questions on the mechanism of immune modulation and activation of regulatory T cells induced by polyvalent IgG.

Questions:
1) How can variable portions of polyvalent IgG (idiotopes or idiotype) induce activation of Treg cells?
2) Can anti-idiotypic T cells act as Treg cells?
3) What is the antigen-specificity of Treg cells activated by polyvalent IgG?
4) Which subpopulation of Treg cells is activated by polyvalent IgG ($CD4^+Foxp3^+$ Treg cells and/or $CD4^+Foxp3^-IL\text{-}10^+$ Treg cells)
5) Is the anti-idiotypic Treg cell-mediated immunomodulation a physiological mechanism working in healthy human subjects to maintain immune tolerance?
6) Could immunization with autologous polyvalent IgG trigger the activation of anti-idiotypic Treg cells, leading to a long-term clinical remission in patients with autoimmune and allergic diseases through the induction of immune tolerance?

IgG, immunoglobulin G; Treg cell, regulatory T cell; Foxp3, forkhead box P3; IL, interleukin.

In a murine model of OVA-immunization to induce OVA-specific IgG antibodies, subcutaneous injections of IVIg induced the activation and expansion of B and $CD4^+$ T cells in the spleen and draining lymph nodes, resulted in a reduction in OVA-specific antibody production, and induced anti-IVIg IgG antibodies that mainly recognized the $F(ab')_2$ fragments of IVIg (44). These immunomodulatory effects could not be induced after subcutaneous injections of two human IgG monoclonal antibodies that share the constant region of IgG but not their idiotypes (44). These observations suggested that the immune responses to idiotypes of IVIg with enormous sequence diversity might produce immunomodulatory effects suppressing the production of anti-OVA IgG antibodies (44). However, in this mouse model, there was no evidence of the activation of Treg cells induced through the subcutaneous injections of IVIg (44). Investigation of the human leukocyte antigen (HLA) class II peptide repertoire from IVIg-loaded human DCs via MHC-associated peptide proteomics (MAPPs) revealed that numerous peptides derived from the hypervariable region of IgG were strongly presented (45). Surprisingly, Tregitopes derived from the constant region of IgG failed to suppress effector immune responses against a specific antigen in both human peripheral blood mononuclear cells and mouse immune cells (45). These results suggested that the immunomodulatory effects of polyvalent IgG were originated from the hypervariable region (idiotype) of IgG and not from the constant region of IgG (Tregiotopes) (Model 2, Figure 1B).

In humans, an author of this review (Nahm DH) reported that the intramuscular administration of autologous total IgG increased the percentage of IL-10-producing $CD4^+$ T cells in the peripheral blood in 13 healthy human subjects (46). However, this report could not provide a detailed molecular mechanism underlying the autologous polyvalent IgG-mediated immunomodulation or the subpopulation of Treg cells activated by autologous polyvalent IgG.

This review provides a summary of results (subpopulation of Treg cells and mechanism) on the polyvalent IgG-induced activation of Treg cells from mouse studies (21, 42, 44, 47–51) (Table 2). Further studies are needed to evaluate the characteristics of polyvalent IgG-derived peptides activating Treg cells and subpopulations of Treg cells mediating polyvalent IgG-induced immunomodulation.

2.2 Hypothetical model 3 and 4: activation of Treg cells by direct binding of polyvalent IgG to surface receptors on T cells or APCs

Addition of polyvalent human IgG to purified $CD4^+CD25^{high}$ T cells from healthy human subjects increased intracellular expression of IL-10 in $CD4^+CD25^{high}$ T cells under experimental cell culture conditions; this observation indicated a direct activation of Treg cells by polyvalent IgG (20). To explain the mechanism underlying polyvalent IgG induced direct activation of T cells, the "hook without bait theory" has been proposed by an author of this review (Victor JR) (52). In this theory, direct binding of idiotype of polyvalent IgG to the TCRs or unidentified antigens on the surface of $CD4^+$ cells can induce differentiation of Treg cells from naive T cells or activate pre-existing Treg cells to secrete IL-10 (Figure 1C). The direct interaction between polyvalent IgG and antigens on the cell membrane of $CD4^+$ thymic T cells induced cytokine production from $CD4^+$ thymic T cells (53). However, this study did not evaluate the activation of Treg cells by direct binding of polyvalent IgG to antigens on the cell membrane of T cells. In a co-culture model of human DCs and $CD4^+$ T cells as well as in a mouse model of experimental autoimmune encephalomyelitis, IVIg was seen to increases Treg cells through the binding of $F(ab')_2$ fragments of IVIg with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) on DCs (54) (Figure 1D). A similar approach demonstrated that polyvalent IgG can specifically interact with murine DC immunoreceptors, favoring the induction of Treg cells (55). Another possibility is the direct binding of polyvalent IgG to an unidentified antigen (or conserved receptor) on the surface of APCs, and this interaction induces the changes of APCs into tolerogenic APCs, promoting the differentiation of naive $CD4^+$ T cells into Treg cells and secretion of IL-10 (Figure 1D).

However, further studies are needed to validate these four hypothetical models of polyvalent IgG-induced Treg cell activation and the subpopulations of Treg cells ($CD4^+Foxp3^+$ T cells and/or Tr1 cells) involved in these processes.

3 Evidence supporting activation of Treg cells by polyvalent IgG in clinical trials

3.1 Evidence supporting activation of Treg cells upon intravenous administration of IVIg in human clinical trials

IVIg has been used as a replacement or immunomodulatory therapy in primary immunodeficiency, secondary immunodeficiency, hematological diseases, neuroimmunological diseases, rheumatic diseases, dermatological diseases, and other conditions including miscarriages (56). Clinical studies on IVIg therapy in primary immunodeficiency patients (57), the treatment of

TABLE 2 Effect of polyvalent IgG-induced regulatory T cell activation in mouse model.

Induced disease	lineage	IgG type	Administration dose (route)	Evaluation timing	Specimen source	Measurement of Treg cell	Immunological effect (or mechanism)	Ref
Experimental autoimmune encephalomyelitis (EAE)	Mouse C57BL/6J female	Human IVIg	0.8 g/kg (IP)	Day 21	Spleen	↑ $CD4^+CD25^+Foxp3^+$	Protection by IVIg was associated with an increase in $CD4^+CD25^+Foxp3^+$ Treg cell numbers and function. IVIg failed to protect against EAE in Treg cell-depleted mice. IVIg had a direct effect on the proliferation of natural Treg cells.	(21)
OVA-induced airway hyperresponsiveness (AHR)	Mouse C57BL/6 male and female	Human IVIg	2 g/kg (day 28) (IP)	Day 32	Lung	↑ $CD4^+CD25^{high}$ $Foxp3^+$	IVIg generated a *de novo* population of peripheral Treg (pTreg) cells in the absence of endogenous Treg cells. IVIg-generated pTreg cells were sufficient for inhibiting OVA-induced AHR. Adoptive transfer of purified IVIg-generated pTreg prior to antigen challenge effectively prevented airway inflammation and AHR in an antigen-specific manner.	(42)
OVA-induced IgE response	Mouse C57Bl/6NCrl female	Human IVIg	1mg, 10mg, 20mg, 50mg (day 0, day7, and day 14) (SC)	Day 15	Spleen LN	No difference of percentage $CD4^+CD25^+$ $Foxp3^+$ between IVIg treatment group and control group	IVIg induced the activation and expansion of B and $CD4^+$ T cells, reduced development of OVA-specific antibody, and induced anti-IVIg IgG antibodies that mainly recognizing the $F(ab')_2$ fragments of IVIg.	(44)
OVA-induced IgE response	Mouse BALB/c male and female	OVA-immunized mouse IgG	600 ug/per pregnant mice at prenatal period (divided in 3 days at days 10,15 and 20) (IV)	Day 20	Spleen	↑ L-10-producing $CD4^+$	Passive transfer of OVA-immunized IgG to pregnant mice increased the frequency of IL-10-producing T cells in offspring and prevented neonatal OVA-sensitization.	(47)
Collagen-induced arthritis (CIA)	Mouse DBA1/J male	Human IVIg	25 mg/mouse at day 7 (IV)	Week 15	Spleen	↑ $CD4^+CD25^+$ $Foxp3^+$ (FACS) ↑ $CD4^+CD25^+$ $Foxp3^+IL\text{-}10^+$ (confocal microscopy)	IVIg increased the number of $Foxp3^+$ Treg cells. The therapeutic effects of IVIg are dependent on IL-10. The treatment effects of IVIG on arthritis were lost in IL-10-knockout mice.	(48)
Herpes simplex virus (HSV) induced fatal encephalitis	Mouse 129S6 male	Human IVIg	3.75 mg/mouse (IP)	Day 1 after HSV infection	Spleen	↑ $CD4^+CD25^+Foxp3^+$	IVIg induced secretion of IL-10 from Treg cells and it produced anti-inflammatory effects that protect against fatal HSV encephalitis.	(49)
OVA-induced airway hyperresponsiveness (AHR)	Mouse C57BL/6	Human IVIg	2 g/kg 24hr before antigen challenge (day 28)(IP)	After 5 days of OVA or sham challenge	Spleen LN Lung	↑ $CD4^+Foxp3^+$	IVIg induced antigen-specific $Foxp3^+$ Treg cells from non-Treg cell precursors. Induction of Treg cells was mediated by tolerogenic dendritic cells.	(50)
Experimental autoimmune encephalomyelitis (EAE)	Mouse C57BL/6J female	Human IVIg	0.8 g/kg/daily from day 0 to day 18 (IP)	Day 9	Spleen	↑ $CD4^+Foxp3^+$	IVIg inhibits the differentiation of naive CD4 T cells into EAE Th1/17 cells and induced an expansion of $Foxp3^+$ Treg cells. $F(ab')_2$ fragments retained this function of IVIg.	(51)

IgG, immunoglobulin G; IgE, immunoglobulin E; Treg cell, regulatory T cell; IVIg, purified polyvalent IgG from the plasma pool of multiple healthy human blood donors; Foxp3, forkhead box P3; OVA, ovalbumin; SC, subcutaneous; IP, Intraperitoneal; IV, intravenous; LN, lymph node; IL, interleukin; FC, fragment crystallizable.

10.3389/fimmu.2023.1242860

autoimmune rheumatic disease (58), Guillain-Barré syndrome (59), eosinophilic granulomatosis (60), Kawasaki disease (61), recurrent miscarriage (62), and repeated implantation failure (63) showed that IVIg therapy increased the percentage of Treg cells (mainly $CD4^+$ $Foxp3^+$ Treg cells) in the peripheral blood (Table 3). In a clinical trial, IVIg therapy in patients with common variable immunodeficiency significantly increased the number of peripheral Treg cells (expressing CD4, CD25, and low levels of CD127) and plasma levels of IL-10 within 30 minutes after the infusion of IVIg, suggesting a highly efficient interaction between polyvalent IgG and Treg cells (64). This review provides a summary of study results (subpopulation of Treg cells and mechanism) on the polyvalent IgG-induced activation of Treg cells in human clinical trials (Table 3) (46, 57–65).

3.2 Clinical application of "active anti-idiotypic therapy" with polyvalent IgG: lessons obtained from clinical trials on intramuscular injection of autologous total IgG in patients with atopic dermatitis

An author (Nahm DH) of this review has tried to develop a new anti-idiotypic therapy through intramuscular injection of autologous total IgG purified from autologous blood via affinity-chromatography using protein A bead in patients with atopic dermatitis (AD) and healthy human subjects (46, 66–70). From clinical trials in patients with AD and healthy human subjects, the novel concept of an "active anti-idiotypic therapy" was developed as described in the following sections.

TABLE 3 Effect of polyvalent IgG-induced regulatory T cell in human clinical trials.

Treatment-target diseases	Study design	IgG type	Administration dose (route)	Evaluation timing	Measurement of Treg cell	Immunological effect (or mechanism)	Ref
Primary immunodeficiency	Prospective uncontrolled	IVIg	0.36-0.72 g/Kg for 1 day (IV)	Pre-infusion *vs.* 7 days after infusion	↑ $CD3^+CD4^+CD25^{High}$ $Foxp3^+$	Anti-inflammatory effects of IVIg inducing expansion of Treg cells was noticed as early as day 7.	(57)
Autoimmune rheumatic disease	Prospective uncontrolled	IVIg	2 g/Kg (IV)	Pre-infusion *vs.* 72 to 96 hour after last infusion	↑ $CD4^+CD25^{high}$	Anti-inflammatory effects of IVIg with enhancement of Treg cells were observed.	(58)
Guillain-Barré syndrome	Prospective controlled	IVIg	0.4 g/Kg/daily for 4 days (IV)	Pre-infusion (0 week) *vs.* 1 week after infusion	↑ $CD4^+Foxp3^+$	IVIg reduced the frequency of Th1 and Th17 cells and expanded Treg cells. IVIg-expanded Treg cells exhibited T cell suppressive function.	(59)
Eosinophilic Granulomatosis	Prospective controlled	IVIg	0.4 g/kg/daily for 5 days (IV)	IVIg *vs.* without IVIg (3, 6, 12 and 24 months after infusion)	↑ $CD25^+$ among $CD4^+$	The frequency of $CD25^+$ cells among $CD4^+$ T cells increased significantly after IVIg treatment.	(60)
Kawasaki disease	Prospective controlled	IVIg	2 g/kg (IV)	Pre-infusion *vs.*48 hour after infusion	↑ $CD4^+CD25^+$ $Foxp3^+$	Percentage of $CD4^+CD25^+Foxp3^+$ cells were significantly increased at 48 hour after IVIg.	(61)
Recurrent miscarriage	Prospective controlled	IVIg	0.4 g/kg every 4 weeks during 32 weeks of gestation (IV)	At the diagnosis of first positive pregnancy *vs.* at week 32 (last infusion of IVIg)	↑ $CD4^+CD25^+$ $CD127^-$	IVIg induced a significant increase of Treg cells.	(62)
Repeated implantation failure	Prospective controlled	IVIg	0.4 g/kg (2 days before embryo transfer) (IV)	2 days before embryo transfer *vs.* 15 days after embryo transfer	↑ $CD4^+$ $Foxp3^+$ $CD127^{-/low}$	IVIg induced a significant increase of Treg cells.	(63)
Common variable immunodeficiency	Prospective controlled	IVIg	0.3 g/kg by slow 2-hour (IV)	30 minutes before infusion *vs.*30 minutes after infusion	↑ $CD4^+$ ↑ $CD4^+CD25^+$ $CD127^{-/low}$	IVIg increased percentage of Treg cells as early as at 30 minutes after IVIg infusion.	(64)
Primary immune thrombocytopenia	Prospective controlled	IVIg	1 g/kg/day for consecutive 2 days (IV)	·Pre-infusion *vs.*2 days after infusion ·Pre-infusion *vs.* week 4 after infusion	↑ $CD4^+CD25^+Foxp3^+$	IVIg induced expansion of Treg cells at day 2 and week 4 after infusion.	(65)
Healthy human subjects	Prospective uncontrolled	Autologous total IgG	0.4 g (8 injection of 50 mg per 4 weeks) (IM)	Pre-injection (0 week) *vs.* weeks 4, 8, and 12	↑ IL-10-producing $CD4^+$ ↑ IFN-γ-producing $CD3^+$	Intramuscular injection of autologous total IgG increased IL-10-producing $CD4^+$ T cells at weeks 4, 8, and 12 compared to baseline (0 week).	(46)
Atopic dermatitis	RCT	Autologous total IgG	0.4 g (8 injection of 50 mg per 7 weeks) (IM)	Pre-injection (0 week) *vs.* week 16	↑ IL-10-producing $CD4^+$	Intramuscular injection of autologous total IgG increased Treg cells at week 16 compared to baseline (0 week).	(66)

IgG, immunoglobulin G; Treg cell, regulatory T cell; IVIg, purified polyvalent IgG from the plasma pool of multiple healthy human blood donors; Foxp3, forkhead box P3; IV, intravenous; IM, intramuscular; IL, interleukin; IFN, interferon; RCT, randomized clinical trial.

3.2.1 Current knowledge on pathogenesis and treatment of atopic dermatitis

AD is an allergic skin disorder characterized by chronic allergic inflammation of the skin with clinical manifestations including itching, dry skin, exudation, and frequently associated with a personal or familial history of allergic diseases (2, 71, 72).

In patients with AD, allergic inflammation of the skin is characterized by the uptake of allergens by APCs that digest allergens and present peptides of the allergen to allergen-specific $CD4^+$ T cells, which secrete Th2 cytokines (IL-4, IL-5, and IL-13) (73). Upon re-exposure, allergens activate Th2 cells to secrete Th2 cytokines, and allergens also bind to IgE antibodies on the mast cells and induce mast cell degranulation and release of chemical mediators, including histamine and other chemo-attractants. These cytokines and chemical mediators induce chronic allergic inflammation (Th2 inflammation) of the skin and clinical symptoms of AD (itching and eczema) (73).

Current topical and systemic immunosuppressants (corticosteroids, cyclosporine, tacrolimus, and methotrexate) nonspecifically suppress inflammatory immune cells and have limited clinical efficacy in patients with moderate-to-severe AD (2). Recently developed monoclonal antibodies to IL-4 receptor alpha or Janus kinase inhibitors suppressing Th2 cell-mediated inflammation demonstrated clinical improvements in a significant number of patients with moderate-to-severe AD (74–77). However, the clinical efficacies of current medical therapies for AD are transient and remain incomplete (71, 78). Therefore, developing a new therapeutic modality modifying the long-term clinical course of AD is needed.

3.2.2 Critical role of decreased number and/or function of Treg cells in the pathogenesis of atopic dermatitis

While Th2 cells may promote allergic inflammation, Treg cells can downregulate Th2 cell function and suppress allergic inflammations. T cell tolerance mediated by Treg cells is the key mechanism underlying immune tolerance in a healthy immune response to self and non-infectious foreign antigens. Immune dysfunction characterized by decreased Treg cell function (reflected by the decreased number and/or function of Treg cells) (79, 80) and excessive activation of Th2 cells producing IL-4 and IL-13 plays a key role in the pathogenesis of AD (2).

Therefore, immunomodulatory therapies to restore Treg cell number and/or function and Th1/Th2-cell balance might be a reasonable approach to induce a long-term treatment-free clinical remission in patients with AD (2, 81).

3.2.3 Immunomodulatory therapies that induced a long-term treatment-free clinical remission in patients with atopic dermatitis

3.2.3.1 Allergen immunotherapy

Allergen immunotherapy is a treatment that involves the repeated administration of sensitized allergens either subcutaneously or sublingually to induce allergen-specific immune tolerance in patients with allergic diseases (82, 83). Allergen immunotherapy is clinically beneficial in AD patients sensitized to house dust mites in a meta-analysis of multiple randomized clinical trials (84, 85). Clinical observational studies reported long-term treatment-free clinical remissions in patients with AD after allergen immunotherapy (86–88). Allergen immunotherapy activates allergen-specific Treg cells to downregulate Th2 cell- and IgE-mediated allergic inflammation. Treg cells are generated during allergen immunotherapy, secrete IL-10, and induce allergen-specific B cells to produce IgG4 antibodies (89). These mechanisms induce tolerance to antigens that reduce allergic symptoms. Induction of peripheral T cell tolerance by Treg cells is the key mechanism involved in allergen immunotherapy (90). Activated Treg cells release IL-10 and suppress Th2 cell-mediated allergic inflammation. However, there are still controversies regarding the main subpopulation of Treg cells mediating therapeutic effects involved in allergen immunotherapy ($CD4^+Foxp3^+$ Treg cells and/or $CD4^+Foxp3^-IL\text{-}10^+$ Treg cells) (91).

3.2.3.2 Natural remission of disease in children with atopic dermatitis

More than 70% of children with AD experience natural remission of their disease before puberty (92); although the mechanism of natural remission of AD in children has not been determined yet, induction of immune tolerance due to activation of Treg cells has been suggested as a mechanism (2). Induction of immune tolerance mimicking the immunological mechanism responsible for the natural clinical remission of AD in children could be an ideal way to achieve clinical remission in patients with AD (2).

3.2.3.3 Intramuscular administration of autologous polyvalent IgG for induction of anti-idiotype immune modulation

An author of this review (Nahm DH) hypothesized that immunizing the human subjects with autologous immunoglobulin might induce Treg cell response with systemic immunomodulatory effects to prevent or treat AD. To evaluate this hypothesis, clinical trials were performed to evaluate the clinical efficacy and immunomodulatory effect of intramuscular administration of autologous polyvalent IgG (total IgG) purified from autologous blood using Protein A bead in patients with AD and healthy human subjects (46, 66–70).

In a randomized controlled trial including 51 patients with AD, 8 weekly intramuscular injections of autologous total IgG (50 mg) for 7 weeks resulted in significant clinical improvements and increased serum levels of IL-10 and interferon (IFN)-γ at week 16 (66). In this study, the percentages of IL-10- and IFN-γ-producing cells in the peripheral blood $CD4^+$ T cells were increased after the intramuscular administration of autologous total IgG in patients with AD (66). These results suggested that intramuscular administration of low doses of autologous total IgG can activate Treg cells and Th1 cells producing IFN-γ and lead to systemic immunomodulatory effects in patients with AD (66).

In a previous clinical study, a long-term clinical improvement for more than nine months was observed in 2 of 3 patients with severe AD who received 8 intramuscular injections of autologous

total IgG 50 mg (total 400 mg) for 4 weeks and were followed up for two years (69). In this study, two of three patients showed clinical improvement for more than 36 weeks after treatment with maximum decreases in clinical severity score greater than 80% from baseline. These two patients showed a long-term (more than 36 weeks) decrease in serum total IgE concentration and peripheral blood eosinophil count with maximum decreases in those values greater than 70% from baseline. No significant side effect was observed during the two years of follow-up period in all three patients (69). This observation suggests a potential of intramuscular administration of autologous total IgG in modifying the long-term disease course of AD.

Changes in T cells before and after intramuscular administration of autologous total IgG were evaluated in 13 healthy human subjects (46). Intramuscular administration of autologous total IgG (8 injections of autologous IgG 50 mg per 4 weeks; total 400 mg IgG) significantly increased the percentage of IL-10-producing $CD4^+$ T cells in peripheral blood $CD4^+$ T cells and the percentage of IFN-γ-producing $CD3^+$ T cells in peripheral blood $CD3^+$ T cells in healthy human subjects (46) (Table 3). These results suggest that intramuscular administration of autologous total IgG can activate Treg cells and IFN-γ-producing T cells in healthy subjects and that it could be a safe and effective method to activate Treg cells in human subjects (46). The significant limitations of these clinical studies are the lack of knowledge on the detailed molecular mechanism underlying Treg cell activation induced by autologous total IgG and the subpopulation of Treg cells ($CD4^+Foxp3^+$ Treg cells and/or $CD4^+Foxp3^-IL\text{-}10^+$ Treg cells) activated by autologous total IgG.

An author of this review (Nahm DH) speculates that the intramuscular administration of autologous total IgG activates pre-existing anti-idiotypic Treg cells that can specifically recognize peptides derived from IgG idiotypes by their TCRs and produce IL-10, based on the observations in healthy human subjects after intramuscular injection of autologous total IgG (46). However, a direct experimental demonstration of the existence or development of anti-idiotypic Treg cells has not been provided yet. Therefore, an author (Nahm DH) of this review proposes that "anti-idiotypic Treg cell" is a missing scientific link that can integrate both "idiotypic network theory" and "Treg cell theory" to explain the mechanism underlying the maintenance of an immune tolerance state (Tables 1, 4).

TABLE 4 Ten future research topics on the detailed mechanism underlying polyvalent IgG-induced activation of regulatory T cells and its clinical application.

Questions for future research topics
1) Which subpopulation of Treg cells responsible for the immunomodulatory effect of polyvalent IgG? (Tr1 cells or $Foxp3^+$ Treg cells)?
2) Can the peptides from idiotypes of the IgG processed by antigen-presenting cells induce a differentiation of naive $CD4^+$ T cells into Treg cells or an activation of pre-existing resting anti-idiotypic Treg cells?
3) Can the direct binding of polyvalent IgG to T cell receptors on the surface of Treg or antigens in the antigen-presenting cells induce activation of Treg cells?
4) Can intramuscular or subcutaneous injection of autologous polyvalent IgG induce activation of Treg cells specific to peptides derived from idiotypes of autologous IgG (anti-idiotypic Treg cells)?
5) Can the existence of anti-idiotypic Treg cell be demonstrated by experiments in cell culture condition or animal models?
6) Can intramuscular or subcutaneous injection of autologous polyvalent IgG increase the production of anti-idiotypic antibodies?
7) Is there any difference in the immunomodulatory effects and clinical efficacy between intravenous injection of polyvalent IgG from healthy blood donors and intramuscular or subcutaneous injection of autologous polyvalent IgG in patients with allergic or autoimmune diseases?
8) Is there a difference in immunomodulatory mechanism induced by intravenous injection of polyvalent IgG from healthy blood donors or intramuscular or subcutaneous injection of autologous polyvalent IgG?
9) Can intramuscular or subcutaneous injection of autologous polyvalent IgG induce long-term clinical improvements in patients with allergic and autoimmune diseases more efficiently compared with intravenous injection of polyvalent IgG from healthy blood donors?
10) Are the concepts of passive anti-idiotype therapy for the explanation of the therapeutic mechanism of intravenous injection of polyvalent IgG from healthy blood donors (supplementing natural anti-idiotypic antibodies to neutralize pathogenic antibodies) and active anti-idiotypic therapy for the induction of anti-idiotypic Treg cell and B cell responses by intramuscular or subcutaneous injection of autologous polyvalent IgG correct?

IgG, immunoglobulin G; Treg cell, regulatory T cell; Tr1 cell, type 1 regulatory T cell; nTreg cell, natural regulatory T cell; Foxp3, forkhead box P3.

3.2.3.4 Comparison of efficacy and mechanism of intravenous injection of heterologous polyvalent IgG and intramuscular injection of autologous total IgG in patients with atopic dermatitis

Expansion of $CD4^+$ $Foxp3^+$ Treg cells in the peripheral blood has been reported in patients with autoimmune diseases after intravenous administration of high-dose (1~2 g/Kg of body weight) heterologous IVIg (60, 65, 93). Continuous regular intravenous infusion of a high dose of polyvalent human IgG is needed to achieve a long-term clinical improvement in patients with autoimmune diseases (60, 91, 93). This requirement of continuous treatment has been attributed to the low concentration and low affinity of natural anti-idiotype antibodies to pathogenic autoantibodies contained in IVIg (17). Clinical trials on high-dose intravenous polyvalent IgG therapy (1~2 g/Kg of body weight per patient) in adult patients with severe AD showed no significant clinical benefit (93). However, intramuscularly administered low doses of purified autologous total IgG (8 intramuscular injections of autologous total IgG 50 mg for 4 weeks) produced potent immunomodulatory effects (increased serum levels of IL-10 and IFN- γ) and long-term clinical improvement compared to intravenous administration of a high dose of heterologous IgG (1~2 g/Kg of body weight per a patient) in adolescent and adult patients with AD (66, 93). These conflicting results from clinical trials on the intravenous administration of heterologous IgG and intramuscular administration of autologous IgG in patients with AD suggest that differences in the origin of polyvalent IgG (heterologous vs. autologous) and route of administration of polyvalent IgG (intravenous vs. intramuscular) can result in different immunomodulatory effects and clinical efficacy (66).

If polyvalent IgG is used as an "antibody" to neutralize pathogenic antibodies (IgG autoantibodies or IgE antibodies), intravenous administration of polyvalent IgG can be the preferred route of administration (66). However, if polyvalent IgG is used as an "antigen" to activate anti-idiotypic Treg cells in patients with allergic and autoimmune diseases, the intramuscular or subcutaneous administration route might be an ideal route of administration. A specific antigen administered through

intravenous injection preferentially interacts with mononuclear cells in the peripheral blood, and a specific antigen administered through intramuscular or subcutaneous injection preferentially interacts with DCs (66).

Intramuscularly administered autologous polyvalent IgG may more efficiently activate pre-existing idiotype-specific Treg cells than with intramuscularly administered heterologous IgG because the pre-existence of idiotype-specific T cell to autologous IgG is an essential prerequisite of the "idiotype-network theory." The potency of activation of Treg cells induced through intramuscular injection of heterologous polyvalent IgG can be weak because of a smaller number of pre-existing idiotype-specific Treg cells than that in case of intramuscular injection of autologous polyvalent IgG.

Further studies are necessary to evaluate the detailed mechanisms underlying systemic immunomodulation induced by intravenous or intramuscular administration of heterologous or autologous human IgG, especially on the mechanism of anti-idiotypic immunomodulation.

3.3 *In vitro* and animal studies about IgG-mediated anti-idiotypic immunomodulation: lessons from experiments using IgG from donors with various immune backgrounds

3.3.1 Immunomodulatory effects induced by polyvalent IgG from donors with various immune backgrounds

In a mouse model of OVA-allergy, the passive transference of polyclonal IgG from allergen-immunized donors to non-immunized pregnant mice increased IL-10-producing $CD4^+$ T cells in the offspring at birth and prevented neonatal OVA-sensitization (47). This effect occurs in the absence of maternal antigen immunization, suggesting that the *in vivo* effect was mediated by idiotypic interactions between the polyvalent IgG from allergen-immunized donors administered to the mother during pregnancy and the fetus lymphocytes (47).

A study suggested that IgG also permeates cells and interacts with intracellular molecules, inhibiting T cell activation after its interaction with nuclear and cytoplasmic components (94). These cell-penetrating antibodies, which could be detected in polyvalent IgG formulations, penetrate various mammalian cell lines and represent ~2% of polyvalent IgG. This possibility also needs to be discussed because it suggests that anti-idiotype IgG may interact with TCRs intracellularly, at an early stage of production of these receptors.

3.3.2 Experimental observations regarding IgG from patients with atopic dermatitis

It has been demonstrated that AD patients can produce detectable levels of anti-IgE autoantibodies in the IgG class (95) and that these antibodies can mediate inflammatory mediators released from basophils and mast cells (96). This observation was confirmed by two studies which showed that the functional activity of IgG anti-IgE autoantibodies from patients with AD induced the release of pro-inflammatory mediators and cytokines from human basophils and mast cells (97, 98).

Human thymic lymphocyte culture with purified IgG showed that pooled IgG from patients with AD could more effectively modulate cytokine production from thymic $CD4^+$ T cells than did polyvalent IgG from healthy human subjects (IVIg) (99). This study demonstrates that IgG from patients with AD induced significantly higher production of IL-17 and IL-10 by intrathymic immature double positive and mature $CD4^+$ T cells than did polyvalent IgG from healthy individuals (99). In a similar *in vitro* experiment, pooled IgG from patients with AD could induce thymic invariant natural killer T cells (CD1d-restricted T cells that employ an invariant TCR alpha chain and a limited repertoire of beta chains) to produce higher levels of intracellular IL-4, IL-10, and IL-17 than did polyvalent IgG from healthy non-atopic individuals (100). The IgG-reactivity toward 1152 human protein fragments was evaluated in 80 individuals (AD patients and healthy controls), and a significant differential IgG-reactivity to four antigens representing keratin-associated protein 17-1 (KRTAP17-1), heat shock protein family A member 4 (HSPA4), S100 calcium-binding proteins A12, and Z (S100A12 and S100Z) was detected (101). The reactivity to these four antigens was more frequent in the patients with severe AD (66%) than in patients with moderate AD (41%) and healthy controls (25%) (101). These observations suggest that purified IgG from patients with AD can modulate T cells more efficiently than does polyvalent IgG from healthy human subjects (IVIg) (99, 100, 102).

4 Six questions on the mechanism underlying polyvalent IgG-induced activation of Treg cells and its clinical application for the treatment of immune diseases

We asked six critical questions regarding the mechanism and its clinical application of polyvalent IgG-induced immune modulation and activation of Treg cells (Table 1). We propose one hypothesis to provide an answer to these six questions regarding the mechanism of polyvalent IgG induced activation of Treg cells and its clinical application (Box 1). These six questions and one hypothesis should be validated in future studies.

In this hypothesis, subcutaneous or intramuscular injection of autologous polyvalent IgG can induce activation of anti-idiotypic Treg cells to downregulate the production of pathogenic antibodies and exaggerated T cell responses harmful to the host and induce an immune tolerance state and ultimately induce a long-term clinical remission in patients with autoimmune and allergic diseases (Box 1). Through the perspective of the idiotypic network theory, immunization with autologous polyvalent IgG might provide a more potent immunomodulatory effects than that provided by heterologous polyvalent IgG in the treatment of autoimmune and allergic diseases.

BOX 1 A new concept of active anti-idiotypic therapy with autologous polyvalent IgG for allergic and autoimmune diseases.

"Subcutaneous or intramuscular administration of autologous polyvalent IgG can activate anti-idiotypic regulatory T cells that can specifically recognize immunogenic peptides generated from idiotypes of autologous IgG by dendritic cells. The activated anti-idiotypic regulatory T cells can induce an immune tolerance state (recovery of immune homeostasis) and a long-term treatment-free clinical remission in patients with allergic and autoimmune diseases."
IgG, immunoglobulin G.

5 Future research directions (research topics) involving the mechanism and clinical application of polyvalent IgG-induced activation of Treg cells

Polyvalent IgG can induce activation of Treg cells to produce IL-10 and exert a systemic immunomodulatory effect and clinical efficacy in patients with allergic and autoimmune diseases. However, this review also exposed a lack of current knowledge regarding the mechanism of polyvalent IgG-induced immunomodulation including Treg cell activation and its role in the development and maintenance of immune tolerance. Therefore, we provide our hypotheses (unproved speculations on the detailed mechanism) to promote further research on this important but neglected research topic by other colleagues. For future investigations, we propose research topics to answer questions on the detailed mechanism and clinical application of polyvalent IgG-induced immunomodulation (Table 4).

6 Conclusion

Experimental studies and clinical trials involving Treg cells suggest that polyvalent IgG acts as a stimulator of Treg cells. However, the mechanism underlying the activation of Treg cells by polyvalent IgG is not defined yet. We hypothesize that anti-idiotypic Treg cells can be activated via intramuscular or subcutaneous injection of autologous polyvalent IgG because idiotypes of polyvalent IgG can act as antigens. The activated anti-idiotypic Treg cells secrete IL-10, and IL-10 suppresses Th2 cell responses to allergens and autoimmune T cell responses to self-antigens and thus can induce a long-term clinical remission of allergic and autoimmune diseases. Further studies are needed to evaluate the detailed molecular mechanism underlying polyvalent IgG-induced Treg cell activation and the clinical usefulness of this immunomodulatory strategy for the treatment of allergic and autoimmune diseases.

Author contributions

All authors contributed in writing and revising the manuscript. DN: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. JV: Funding acquisition, Writing – original draft, Writing – review & editing. All authors contributed to the article and approved the submitted version.

Funding

The authors declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1D1A1B07045762), by the Laboratory of Medical Investigation-56, Medical School, University of São Paulo, São Paulo, Brazil (LIM-56 HC-FMUSP), by the National Council for Scientific and Technological Development (CNPq) grant# 302937/2021-8, and by the São Paulo Research Foundation (FAPESP) grant #2021/08225-8.

Acknowledgments

This collaborative work was started by DN's initial email contact and suggestion to JV.

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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 18 December 2023
DOI 10.3389/fimmu.2023.1298938

OPEN ACCESS

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RECEIVED 02 October 2023
ACCEPTED 27 November 2023
PUBLISHED 18 December 2023

CITATION
Yilmazer A, Zevla DM, Malmkvist R, Rodríguez CAB, Undurraga P, Kirgin E, Boernert M, Voehringer D, Kershaw O, Schlenner S and Kretschmer K (2023) Selective ablation of thymic and peripheral Foxp3$^+$ regulatory T cell development. *Front. Immunol.* 14:1298938. doi: 10.3389/fimmu.2023.1298938

Selective ablation of thymic and peripheral Foxp3$^+$ regulatory T cell development

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Foxp3$^+$ regulatory T (Treg) cells of thymic (tTreg) and peripheral (pTreg) developmental origin are thought to synergistically act to ensure immune homeostasis, with self-reactive tTreg cells primarily constraining autoimmune responses. Here we exploited a Foxp3-dependent reporter with thymus-specific GFP/Cre activity to selectively ablate either tTreg (ΔtTreg) or pTreg (ΔpTreg) cell development, while sparing the respective sister populations. We found that, in contrast to the tTreg cell behavior in ΔpTreg mice, pTreg cells acquired a highly activated suppressor phenotype and replenished the Treg cell pool of ΔtTreg mice on a non-autoimmune C57BL/6 background. Despite the absence of tTreg cells, pTreg cells prevented early mortality and fatal autoimmunity commonly observed in Foxp3-deficient models of complete Treg cell deficiency, and largely maintained immune tolerance even as the ΔtTreg mice aged. However, only two generations of backcrossing to the autoimmune-prone non-obese diabetic (NOD) background were sufficient to cause severe disease lethality associated with different, partially overlapping patterns of organ-specific autoimmunity. This included a particularly severe form of autoimmune diabetes characterized by an early onset and abrogation of the sex bias usually observed in the NOD mouse model of human type 1 diabetes. Genetic association studies further allowed us to define a small set of autoimmune risk loci sufficient to promote β cell autoimmunity, including genes known to impinge on Treg cell biology. Overall, these studies show an unexpectedly high functional adaptability of pTreg cells, emphasizing their important role as mediators of bystander effects to ensure self-tolerance.

KEYWORDS

immune tolerance, autoimmunity, scurfy, diabetes, T cell development, pTreg, tTreg, Foxp3

Introduction

The discovery of genetic *Foxp3* gene mutations as the culprit of the fatal autoimmune syndrome in the spontaneous *scurfy* mouse mutant (1, 2) and human IPEX patients (3, 4) provided the basis for unraveling the key role of Foxp3$^+$ regulatory T (Treg) cells in dominant immunological tolerance. Observations in *Foxp3* gene-targeted mice further corroborated Treg cell paucity as the primary cause of early death and multi-organ autoimmunity in Foxp3-deficient mice (5) but also revealed the peripheral accumulation of Treg cell-like 'wanna-be' CD4$^+$ T cells with self-reactive specificities (6–8) that contribute to the disease pathology (9, 10). Acute Foxp3$^+$ Treg cell ablation recapitulated some, but not all aspects of the *scurfy* syndrome in non-autoimmune-prone mice (11, 12) and highlighted the continuous requirement of Treg cells to constrain organ-specific autoimmune responses in the spontaneous non-obese diabetic (NOD) mouse model of human type 1 diabetes (T1D) (13).

Since then, it has become clear that the physiologic Treg cell pool is developmentally heterogeneous (14–16), consisting of intrathymically (tTreg) and peripherally (pTreg) induced Treg cells that originate from distinct CD4$^+$CD25^{high}Foxp3$^-$ precursor cells residing in thymus (17) and peripheral lymphoid tissues (18), respectively. In the thymus, distinct CD4$^+$CD8$^-$ single-positive (CD4SP) precursor cells, which exhibit low levels of Foxp3 protein preceding the up-regulation of CD25 expression, further expand the mature tTreg cell repertoire (19). In early studies examining the functional specialization of Treg cell developmental subsets by adoptive transfer immunotherapy of newborn *scurfy* mice (20), total Foxp3$^+$ Treg cells prevented disease lethality, but did not suppress chronic inflammation and autoimmunity, which required the provision of Foxp3-sufficient CD4$^+$ T cells to facilitate the extrathymic conversion of initially Foxp3$^-$ T cells into functional Foxp3$^+$ Treg cells (20). According to the prevailing view, tTreg cells are primarily positively selected by self-antigens during intrathymic development and are functionally specialized to control immune homeostasis and autoimmune responses (14, 21). The tTreg cell compartment in the spleen (SPL) and lymph nodes (LNs) has also been proposed to harbor Foxp3$^+$ST2$^+$ common precursors for tissue-type Treg cells (22) that accumulate and perform homeostatic and regenerative functions in nonlymphoid tissues (23), such as the visceral adipose tissue (24, 25). Consistent with tTreg cells as primary regulators of autoimmune responses, studies in mice with *Foxp3* gene-targeted deletion of conserved non-coding region 1 (CNS1) (Foxp3.CNS1$^{-/-}$), which exhibit a significant, albeit incomplete block of pTreg cell development (26), failed to reveal severe autoimmune symptoms and have implicated pTreg cells in the control of immune responses at mucosal surfaces (27) and maternal-fetal tolerance (28). More recently, pTreg cells dependent on the gut microbiota have been shown to mediate functions beyond dominant suppression by facilitating muscle regeneration (29). With regard to a putative role of pTreg cells in the control of autoimmune responses, previous studies in the NOD model showed that dendritic cell (DC)-targeted self-antigen can encourage highly diabetogenic CD4$^+$Foxp3$^-$ T cells to acquire a Foxp3$^+$ pTreg cell phenotype (30, 31), and that naturally induced, β cell-reactive pTreg cells are superior to tTreg cells with the same T cell receptor (TCR) specificity in constraining the manifestation of overt diabetes in a NOD.*Rag1*$^{-/-}$ adoptive transfer model (32). While Foxp3-deficient NOD mice failed to develop insulitis and overt diabetes (33), studies in Foxp3.CNS1$^{-/-}$ NOD mice have provided ambiguous results, providing evidence for either a dispensable (34) or nonredundant function (35) of Foxp3.CNS1-dependent pTreg cells in the control of destructive β cell autoimmunity. In these studies, the relative contribution of tTreg cells to autoimmune β cell protection has not been directly addressed, owing to the lack of mouse models with selective tTreg cell paucity.

Here, we have exploited tTreg cell lineage-specific GFP/Cre recombinase activity in dual Foxp3$^{RFP/GFP}$ reporter mice (32, 36) to generate complementary mouse lines that are deficient in either the tTreg (37) or pTreg cell lineage, while sparing the respective sister population. The results of subsequent loss-of-function studies revealed an unexpectedly high functional adaptability of naturally occurring pTreg cells in mice with selective tTreg cell paucity, thereby preventing the manifestation of severe *scurfy*-like symptoms commonly observed in mice with complete Treg cell deficiency. However, the acquisition of an increased genetic autoimmune risk associated with compromised Treg cell activity unleashed high mortality and a distinct pattern of autoimmune diseases, including severe β cell autoimmunity and overt diabetes.

Materials and methods

Selective *in vivo* ablation of developmental Treg cell sublineages

Foxp3$^{RFP/GFP}$ mice (Figure 1A) (32), congenic CD45.1 *scurfy* mice, and Rag2$^{-/-}$ mice were on the C57BL/6 (B6) background. NOD.Foxp3$^{RFP/GFP}$ mice were obtained by backcrossing B6.Foxp3$^{RFP/GFP}$ mice onto the NOD/ShiLtJ background (Jackson Laboratories, Bar Harbor, USA) for ≥ 14 generations (32). For tTreg cell ablation (Figure 1B), B6.R26^{DTA} mice with Cre-activatable diphtheria toxin A (DTA) expression from the ubiquitous *Rosa26* gene locus (38) were crossed with B6.Foxp3$^{RFP/GFP}$ mice (32, 37) or backcrossed to NOD.Foxp3$^{RFP/GFP}$ mice, as indicated. For pTreg cell ablation (Figure 1C), a conditional Foxp3-STOP allele with Cre-activatable Foxp3 expression was crossed to B6.Foxp3$^{RFP/GFP}$ mice, or backcrossed to NOD.Foxp3$^{RFP/GFP}$ mice, as indicated. The Foxp3-STOP allele was developed at the University of Leuven (Genome Engineering Platform). In brief, a transcriptional STOP cassette, consisting of two loxP-flanked SV40 polyadenylation sites, was introduced by conventional gene targeting in E14 ES cells between exon 4 and 5 of the *Foxp3* gene, followed by excision of an FRT-flanked neomycin resistance gene using deleter mice [*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J; Stock No. 003946] (39). The conditional Foxp3-STOP allele was then backcrossed onto the B6 background for ≥ 10 generations and crossed with B6.Rag2$^{-/-}$ mice, protecting B6.Foxp3-STOP mice from severe autoimmunity due to complete Foxp3$^+$ Treg cell deficiency. All NOD mouse lines were fed with NIH #31M rodent diet (Altromin, Germany), and their blood glucose levels were routinely determined once a week using whole blood from the tail vein and Accu-Chek® Aviva (Roche).

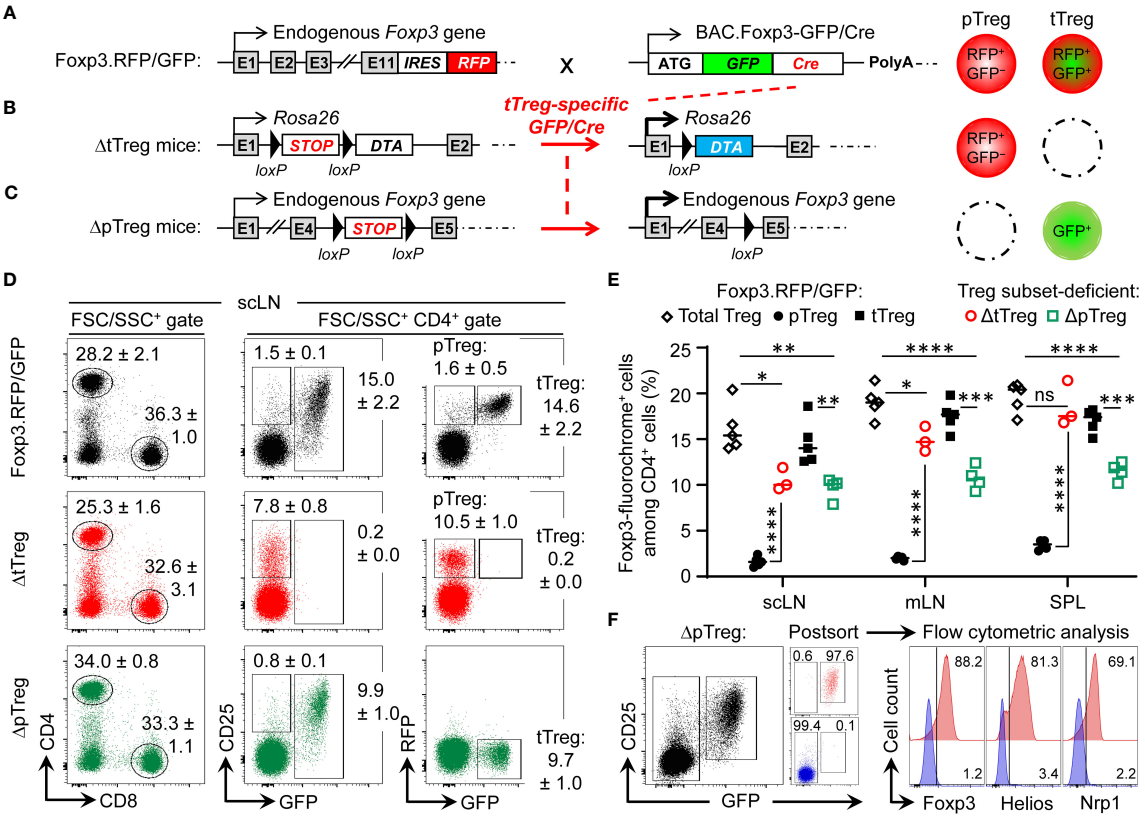

FIGURE 1
Selective ablation of tTreg and pTreg cell development *in vivo*. **(A–C)** Schematic overview of genetic strategy. **(A)** Foxp3$^{RFP/GFP}$ mice. RFP is expressed from an IRES downstream of the endogenous *Foxp3* gene in both pTreg and tTreg cells. Restricted activation of BAC.Foxp3$^{GFP/Cre}$ reporter expression to the thymus results in tTreg cell lineage-specific GFP/Cre activity and induction of gene expression by loxP-flanked STOP cassette excision. **(B)** ΔtTreg mice. Ablation of tTreg cells by GFP/Cre-mediated induction of diphtheria toxin A (DTA) expression. **(C)** ΔpTreg mice. A Foxp3.STOP cassette precludes pTreg cell development, while tTreg cell development can proceed after GFP/Cre-mediated induction of endogenous *Foxp3* gene expression. For this, the Foxp3$^{IRES-RFP}$ reporter in **(A)** was replaced by a Cre-activatable Foxp3.STOP cassette. **(D–F)** Flow cytometry of Treg cells in peripheral lymphoid tissues. **(D)** Representative dot plots of (left) FSC/SSC-gated and (middle, right) CD4-gated cells from subcutaneous lymph nodes (scLNs) of 21-22-week-old males of indicated mouse lines. Numbers in dot plots represent mean percentages of cells ± SD within the respective gate. **(E)** Percentages of CD4-gated Foxp3-fluorochrome$^+$ Treg cells in scLNs, mesenteric LNs (mLNs), and spleen (SPL) of Foxp3$^{RFP/GFP}$ mice (pTreg: filled black circles, n = 5; tTreg: filled black squares, n = 5), ΔtTreg mice (pTreg: open red circles, n = 3), and ΔpTreg mice (tTreg: open green squares, n = 4). Note that the corresponding cell numbers are shown in Supplementary Figure S1. Symbols and horizontal lines represent individual mice and mean values, respectively. Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001. **(F)** CD4$^+$GFP$^-$ T cells and CD4$^+$CD25$^+$GFP$^+$ tTreg cells were FACS-purified from peripheral lymphoid organs of 3-5 males at 10 weeks of age and subjected to flow cytometric analysis of Foxp3, Helios, and Nrp1 expression after intracellular staining using fluorochrome-conjugated mAbs. Numbers in dot plots and histograms represent the percentage of cells within the respective gate.

Mice were considered diabetic at blood glucose levels above 200 mg/dl on at least two consecutive measurements or with blood glucose levels once above 400 mg/dl. All mice were housed and bred at the Animal Facility of the CRTD under specific pathogen-free conditions. Animal experiments were performed as approved by the Landesdirektion Dresden (25-5131/502/5, TVA 5/2020; 25-5131/522/43, TVV41/2021).

Histopathology

After euthanizing the mice using CO_2 inhalation, organs were collected and briefly washed in PBS. Subsequently, the tissues were fixed in a 4% paraformaldehyde solution (Sigma-Aldrich), paraffin-embedded, and 5 µm sections were cut. These sections were then stained with hematoxylin and eosin to assess histopathological changes. A total of 13 organs were examined (lung, heart, thymus, thyroid gland, stomach, liver, intestine, kidney, pancreas, urinary bladder, mesenteric adipose tissue, reproductive tract, brain) in a blinded manner to evaluate the presence and extent of inflammation and necrosis (none: 0; mild: +; moderate: ++; severe: +++) as described elsewhere (40).

Flow cytometry and cell sorting

All single cell suspensions were prepared in Hank's buffer (1×HBSS, 5% FCS, 10mM HEPES; all ThermoFisher, Life Technologies). For this, thymus (THY), spleen (SPL), mesenteric lymph nodes (mLN), pancreatic LN (pLN), and a pool of subcutaneous LN (scLN) (*Lnn. mandibularis, Lnn. cervicales superficiales, Lnn. axillares et cubiti, Lnn. inguinales superficiales,*

and *Lnn. subiliaci*) were meshed through 70 µm cell strainers (BD Biosciences). Bone marrow (BM) cells were harvested from femurs and tibias by flushing mechanically dissociated bones or intact bone cavities with Hank's buffer, followed by filtration through 70 µm cell strainers (BD). Single cell suspensions from SPL and BM were subjected to red blood cell lysis (erythrocyte lysis buffer, EL; Qiagen). Monoclonal antibodies (mAbs) to B220 (RA3-6B2), CD3ϵ (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD25 (PC61), CD62L (MEL-14), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD103 (M290), c-Kit (2B8), GITR (DTA-1), ICOS (7E.17G9), KLRG1 (2F1), PD-1 (29F.1A12), ST2 (U29-93), IgD (11-26c), IgM (II/41), MHC class II (I-A^b: M5/114.15.2; I-A^{G7}: OX-6), Foxp3 (FJK-16s), Helios (22F6), IL-10 (JES5-16E3), IFN-γ (XMG1.2), IL-17a (eBio17B7), IL-2 (JES6-5H4), IL-4 (11B11), IL-5 (TRFK5), TNF (MP6-XT22), Fc receptor-blocking mAb against CD16/32 (93), and fluorochrome-conjugated streptavidin (BUV395, eFlour450, APC and PE-Cy7) were purchased from BD, eBioscience, or Biolegend. Abs to Nrp1 (polyclonal goat IgG-AF700) were purchased from R&D Systems. Intracellular expression of cytokines and transcription factors was analyzed using the respective fluorochrome-coupled mAbs in conjunction with either the BD Cytofix/Cytoperm kit (BD) or the Foxp3 staining buffer set (eBioscience) according to the manufacturer's protocol. The numbers of viable cells were determined using propidium iodide and a MACSQuant (Miltenyi Biotec). Before cell sorting, cells were enriched for $CD4^+$ or $CD25^+$ cells using biotinylated mAbs directed against CD4 or CD25, respectively, streptavidin-conjugated microbeads, fluorochrome-conjugated streptavidin, and the AutoMACS Pro magnetic cell separation system (Miltenyi Biotec). Samples were stained with DRAQ7 (BioStatus) for dead cell exclusion, filtered through 40 µm cell strainers, and analyzed on a LSR Fortessa or sorted using a FACS Aria II or III (all BD). Data were analyzed using FlowJo software (Version 10.8.1, Tree Star Inc.).

Adoptive T cell transfer

Single cell suspensions from pooled LNs and SPL of B6.$Foxp3^{RFP/GFP}$ donors (CD45.2) were subjected to CD4-based magnetic bead enrichment, followed by FACS-based isolation of total $CD4^+$ T cells (i.e., including GFP^+ tTreg cells) and $CD4^+GFP^-$ T cells (i.e., depleted of GFP^+ tTreg cells). 1 x 10^7 cells were injected i.p. into ≤ 2-day-old congenic CD45.1 *scurfy* recipient mice.

T cell culture

T cells were cultured in 96-well round-bottom plates (Greiner) at 37°C and 5% CO_2 in 200 µl RPMI complete medium [RPMI 1680 medium supplemented with 1 mM Sodium pyruvate, 1 mM HEPES, 2 mM Glutamax, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 100 µg/ml Gentamycin, 0.1 mM non-essential amino acids, 0.55 mM β-mercaptoethanol and 10% FCS (v/v); all ThermoFisher, Life Technologies]. Prior to the flow cytometric analysis of intracellular cytokines, single cell suspensions were stimulated for 4 h in RPMI complete medium, using 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 200 ng/ml ionomycin (Iono), in the presence of 20 µg/ml Brefeldin A (all Merck, Sigma-Aldrich). For *in vitro* suppression, $CD4^+CD62L^{high}CD25^-Foxp3^-$ T responder (Tresp) cells and $CD4^+CD25^+Foxp3^+$ Treg cells (RFP^+GFP^+ tTreg or RFP^+GFP^- pTreg cells from B6.$Foxp3^{RFP/GFP}$ mice; and GFP^+ tTreg and RFP^+GFP^- pTreg cells from B6 mice with selective pTreg and tTreg cell paucity, respectively) were FACS-isolated from peripheral lymphoid tissues. After labeling with the cell proliferation dye eFluor670 (5 µM, eBioscience), 5 × 10^4 Tresp cells were cultured in triplicate wells with 10^5 T cell-depleted splenocytes (magnetic bead depletion using anti-CD3, -CD4, and -CD8 mAb) and soluble anti-CD3ϵ mAb (0.5 µg/ml), either alone or with Treg cells at different Treg : Tresp cell ratios, as indicated. On day 3 after initiation of cultures, Tresp cell proliferation and CD25 expression was assessed by flow cytometry.

Genomic PCR-based *Idd* gene analysis

Genomic DNA was isolated from tail biopsies using the NucleoSpin DNA RapidLyse kit (Macherey-Nagel) according to the manufacturer's protocol. Genomic PCR was performed using DreamTag green DNA polymerase and buffer, dNTPs (all Thermo Fisher, Life Technologies), a set of 36 primer pairs (Eurofins Genomics) that cover most of the known *Idd* loci (see Supplementary Materials and Methods for a complete list) (41), and a Biometra Trio Thermocycler (Analytik Jena).

Statistical analysis

Statistical significance was assessed using Prism 8 software (Version 8.4.3, GraphPad Software Inc., CA, USA). As indicated, the Student's *t-test* (unpaired, two-tailed), Long-rank test (multiple comparisons with Bonferroni correction), and Chi-square test was used to assess statistical significance. Differences were considered as significant when *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Results

tTreg-specific Cre activity enables selective blockage of tTreg and pTreg cell development

In dual $Foxp3^{RFP/GFP}$ reporter mice (Figure 1A), expression of the GFP/Cre fusion protein closely correlates with Foxp3 protein expression in the tTreg cell lineage, as transcriptional activation of the BAC.$Foxp3^{GFP/Cre}$ reporter is restricted to the thymic *in vivo* environment (32, 36, 37, 42). In brief, the BAC.$Foxp3^{GFP/Cre}$ reporter was completely inactive in physiologic $Foxp3^-CD4^+CD25^+$ pTreg cell precursors at peripheral sites (18, 32), in various experimental settings of pTreg cell induction *in vivo*, and upon artificial Foxp3 induction in naïve $CD4^+Foxp3^-$ T cells *in vitro* (32). Accordingly, thymic $Foxp3^-CD25^+$ CD4SP tTreg cell precursors upregulated Foxp3-driven GFP/Cre expression during developmental

progression *in situ*, or after intrathymic injection, but not *in vitro* in IL-2-supplemented cultures (32).

In Foxp3$^{RFP/GFP}$ x R26^{DTA} mice (hereafter referred to as ΔtTreg mice) (37), GFP/Cre recombinase activity induces DTA expression selectively in the tTreg cell lineage by excision of an upstream loxP-flanked STOP cassette (38) (Figure 1B). DTA-mediated ablation (≥ 99.8%) resulted in the absence of RFP$^+$GFP$^+$ tTreg cells in scLN (Figure 1D) and at other peripheral sites, such as mesenteric LN and SPL (Figure 1E). A similarly high ablation efficiency was observed in peripheral lymphoid tissues of ΔtTreg mice with heterozygous (B6.R26^{DTA}) and homozygous (B6.R26$^{DTA/DTA}$) expression of the R26.STOP-DTA transgene, and over a wide age range (3-80 weeks; data not shown). As compared to Foxp3$^{RFP/GFP}$ mice, selective tTreg cell paucity had no appreciable impact on the proportional distribution of CD4$^+$ and CD8$^+$ T cells in scLNs (Figure 1D, left), but was accompanied by a > 6-fold increase in the percentage of RFP$^+$GFP$^-$ pTreg cells among CD4-gated T cells (Foxp3$^{RFP/GFP}$ mice: 1.6 ± 0.5%; ΔtTreg mice: 10.5 ± 1.0%) (Figures 1D, E; see Supplementary Figure S1 for CD4$^+$ T cell and Treg cell numbers). This marked increase in the pTreg cell population size of ΔtTreg mice largely compensated for the numerical impairment of the overall Treg cell pool in peripheral lymphoid tissues (Figure 1E; Supplementary Figure S2A), but to a significantly lesser extent in peripheral blood (Supplementary Figure S2B). In aged, > 1-year-old ΔtTreg mice, pTreg cells even exceeded total Treg cell frequencies of age-matched Foxp3$^{RFP/GFP}$ mice (Supplementary Figure S2C).

To obtain mice with selective pTreg cell paucity (hereafter referred to as ΔpTreg mice), the Foxp3$^{IRES-RFP}$ reporter of Foxp3$^{RFP/GFP}$ mice was replaced by breeding with a Cre-activatable Foxp3.STOP cassette (Figure 1C). Hemizygous and homozygous Foxp3.STOP mice succumb to severe *scurfy* disease due to the absence of functional Foxp3 protein and Treg cells (43). In ΔpTreg mice, BAC.Foxp3$^{GFP/Cre}$-mediated activation of Foxp3 expression selectively in tTreg cell lineage-committed thymocytes allowed for the formation of a robust peripheral GFP$^+$ tTreg cell compartment (Figures 1D, E; Supplementary Figure S1B), while the extrathymic generation of pTreg cells remained precluded by the Foxp3.STOP cassette. Consistently, in peripheral lymphoid tissues of ΔpTreg mice, the expression of Foxp3, Helios, and Nrp1 was absent in FACS-purified CD4$^+$GFP$^-$ cells, but readily detectable in CD4$^+$GFP$^+$ tTreg cells (Figure 1F). Interestingly, selective pTreg cell deficiency resulted in a sustained reduction of the peripheral tTreg cell pool in adult (Figure 1E; Supplementary Figure S2A) and aged (Supplementary Figure S2C) ΔpTreg mice, consistent with previous observations in Foxp3.CNS1$^{-/-}$ mice with impaired pTreg cell development (26, 27). These data, in conjunction with the ability of pTreg cells to compensate for the tTreg cell loss in ΔtTreg mice, suggest that pTreg cells are subject to less stringent constraints of the T cell receptor (TCR)-dependent clonal niche in peripheral lymphoid tissues, as compared to tTreg cells (44–46).

Lymphopoiesis in ΔpTreg and ΔtTreg mice

Acute and chronic inflammatory immune responses are well-known to modulate hematopoietic activity, as exemplified by the manifestation of severe lympho-hematopoietic defects during ontogeny of *scurfy* mice (47–50). In the *scurfy* model, autoimmune-mediated thymic aberrations include severe post-developmental atrophy associated with enhanced apoptosis of CD4$^+$CD8$^+$ double-positive (DP) cells and concomitantly increased frequencies of CD4SP and CD8$^+$ SP (CD8SP) cells (50). Unexpectedly, the proportional distribution of DP and SP cells in young (3-4-week-old; data not shown) and adult (13-22-week-old) (Figure 2A) ΔtTreg mice did not significantly differ from age-matched cohorts of ΔpTreg mice and Treg cell-proficient Foxp3$^{RFP/GFP}$ mice. The average thymus size of adult ΔtTreg mice was only moderately reduced (< 2-fold; Figures 2B, C). We occasionally observed rare cases (≤ 10%) of severe thymic atrophy, which were restricted to individual ΔtTreg mice (Figures 2B, C) that additionally exhibited a markedly reduced body weight (ΔtTreg: mouse #1, 11.4 g; mouse #2-5, 28.4 ± 2.8 g; WT: 27.9 ± 1.4 g; ΔpTreg: 31.6 ± 4.1 g), while high frequencies of pTreg cells in peripheral lymphoid tissues remained unaffected (data not shown).

Next, we extended our observation of normal T lymphopoiesis in the majority of ΔtTreg mice to the analysis of B lymphopoiesis in the adult bone marrow (BM). In BM of *scurfy* mice, T cell-mediated autoimmune responses and systemically elevated levels of inflammatory cytokines have been shown to cause a complete block of B cell development, which is reflected by the absence of early Pro/Pre-B-I cell precursors and newly formed IgM$^+$ B cells (47–50). However, our comparative flow cytometric analyses failed to reveal evidence for dysregulated B lymphopoiesis in ΔtTreg and ΔpTreg mice, as compared to Treg cell-proficient Foxp3$^{RFP/GFP}$ mice (Supplementary Figure S3). This included comparable BM and SPL cellularity (Supplementary Figure S3A), as well as proportions and numbers of early B220$^+$c-kit$^+$ Pro/Pre-B-I precursor cells and immature B220^{low}IgM$^+$ B cells in BM (Supplementary Figures S3B, C), and of newly formed IgD^{low}IgM^{high} B cells in the SPL (Supplementary Figures S3D, E).

DTA-mediated tTreg cell ablation occurs prior to thymic exit

In the thymus of Foxp3$^{RFP/GFP}$ reporter mice, tTreg cell lineage commitment induces the sequential expression of RFP and GFP in initially Foxp3$^-$CD25$^+$ CD4SP cells (32, 37). Specifically, the developmental progression of Foxp3$^-$CD25$^+$ CD4SP cells first initiates the simultaneous up-regulation of Foxp3 and RFP protein (giving rise to CD25$^+$RFP$^+$GFP$^-$ cells) (Figure 2D; left panel), which is then followed by the timely delayed up-regulation of GFP/Cre expression, giving rise to newly formed Foxp3$^+$CD25$^+$ tTreg cells that are RFP$^+$GFP$^+$ (Figure 2E; left panel). In ΔpTreg mice, flow cytometry revealed no adverse effects of selective pTreg cell paucity on tTreg cell development (Figures 2D, E; right panels) and numbers of newly formed Foxp3$^+$ tTreg cells (Figure 2F). In the thymus of ΔtTreg mice, tTreg cell development proceeded to the CD25$^+$RFP$^+$GFP$^-$ CD4SP stage (Figure 2D; middle panel), but subsequent up-regulation of BAC.Foxp3$^{GFP/Cre}$ reporter expression promoted DTA-mediated induction of apoptosis prior to thymic exit of newly formed

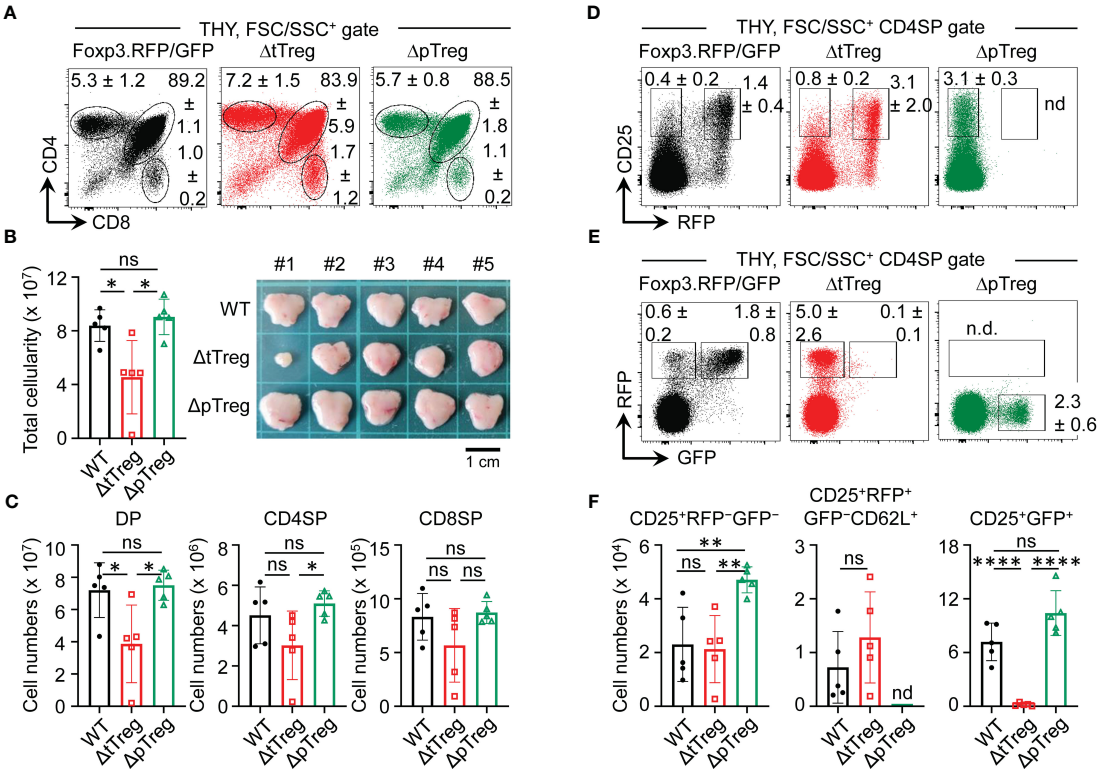

FIGURE 2
Thymopoiesis in adult ΔtTreg and ΔpTreg mice. Flow cytometry of thymic T cell development in Foxp3$^{RFP/GFP}$, ΔtTreg, and ΔpTreg mice. **(A–C)** T cell development. **(A)** Representative flow cytometry of CD4 and CD8 expression among FSC/SSC-gated cells from the thymus (THY) of 17-22-week-old males, as indicated. **(B)** Total thymic cellularity (left) and thymus size (right). **(C)** Numbers of DP, CD4SP, and CD8SP cells. **(D–F)** tTreg cell development. Representative flow cytometry of **(D)** CD25 and Foxp3^{IRES}-driven RFP expression, and **(E)** RFP and BAC. Foxp3$^{GFP/Cre}$-driven GFP expression among gated CD4SP cells, as depicted in **(A)**. **(F)** Numbers of CD25$^+$RFP$^-$GFP$^-$ (left), CD25$^+$RFP$^+$GFP$^-$ (middle, pre-gated on CD62L$^+$ cells to exclude mature recirculating CD62L$^-$ Treg cells) and CD25$^+$GFP$^+$ (right) thymocytes. Note that ΔpTreg mice lack the Foxp3$^{IRES\text{-}RFP}$ reporter (n.d., not detectable). Numbers in dot plots in **(A, D, E)** represent mean percentages of cells ± SD within the respective gate. Symbols and bars in **(B, C, F)** represent individual mice and mean values ± SD, respectively. Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. Data are from a single experiment (5 mice per group) representative of 4 experiments performed (3-6 mice per experiment).

CD25$^+$RFP$^+$GFP$^+$ tTreg cells (Figure 2E; middle panel; Figure 2F). Thus, the observed deficiency in RFP$^+$GFP$^+$ tTreg cells at peripheral sites (Figures 1D, E) was already established within the thymus. Whereas, the proportional increase of CD4-gated RFP$^+$GFP$^-$ cells in ΔtTreg mice (Figures 2D, E) could be attributed to the intrathymic accumulation of mature pTreg cells (Supplementary Figure S4A) that originated from peripheral lymphoid tissues recirculating to the thymus, as indicated by heterogeneous CD25 expression levels and a 'recirculating' CD62L$^-$CD69$^+$CD44^{high} phenotype (Supplementary Figure S4B) (51, 52).

pTreg cells prevent the early manifestation of severe autoimmunity in ΔtTreg mice

In our B6 *scurfy* colony maintained under specific-pathogen-free (SPF) conditions, approximately 50% of mice succumb to premature death by 35 days of age due to fatal autoimmunity associated with Foxp3 deficiency, and no mice live beyond 50 days (50). Within 35 days after birth, ΔtTreg (Figure 3A) and ΔpTreg (Figure 3B) mice appeared overall healthy, showing no appreciable spontaneous mortality (Figures 3A, B) or other *scurfy*-like symptoms (scaliness and crusting of eyelids/ears/tail, hepatomegaly, splenomegaly, lymphadenopathy, *etc.*) (data not shown). Consistently, our analysis of genotype distribution among 5-week-old mice revealed an unbiased heredity of the ΔtTreg (Figure 3C) and ΔpTreg (Figure 3D) phenotype.

The impaired generation of pTreg cells in Foxp3.CNS1$^{-/-}$ mice has been reported to impinge on maternal-fetal tolerance by increasing the resorption of semiallogeneic fetuses (28). Our data show that the number of viable offspring produced by syngeneic ΔtTreg pair mating did not significantly differ from that of Foxp3$^{RFP/GFP}$ mice (Figure 3E). In contrast, interstrain breeding of ΔpTreg mice gave rise to significantly reduced numbers of offspring, correlating with the ΔpTreg phenotype of the breeding female (Figure 3F). These findings in ΔtTreg and ΔpTreg mice, in conjunction with impaired implantation of syngeneic embryos after maternal Foxp3$^+$ Treg cell depletion (53, 54), imply that pTreg cells may contribute to maternal-fetal tolerance even in syngeneic pregnancy.

The unexpected absence of severe *scurfy*-like symptoms in young B6.ΔtTreg mice suggested that selective tTreg cell paucity can be largely compensated by increased pTreg cell numbers (Figures 1D, E). We next asked whether the observed pTreg cell behavior in the ΔtTreg model can be recapitulated in *scurfy* mice neonatally injected with CD4$^+$GFP$^-$ T cell populations (including

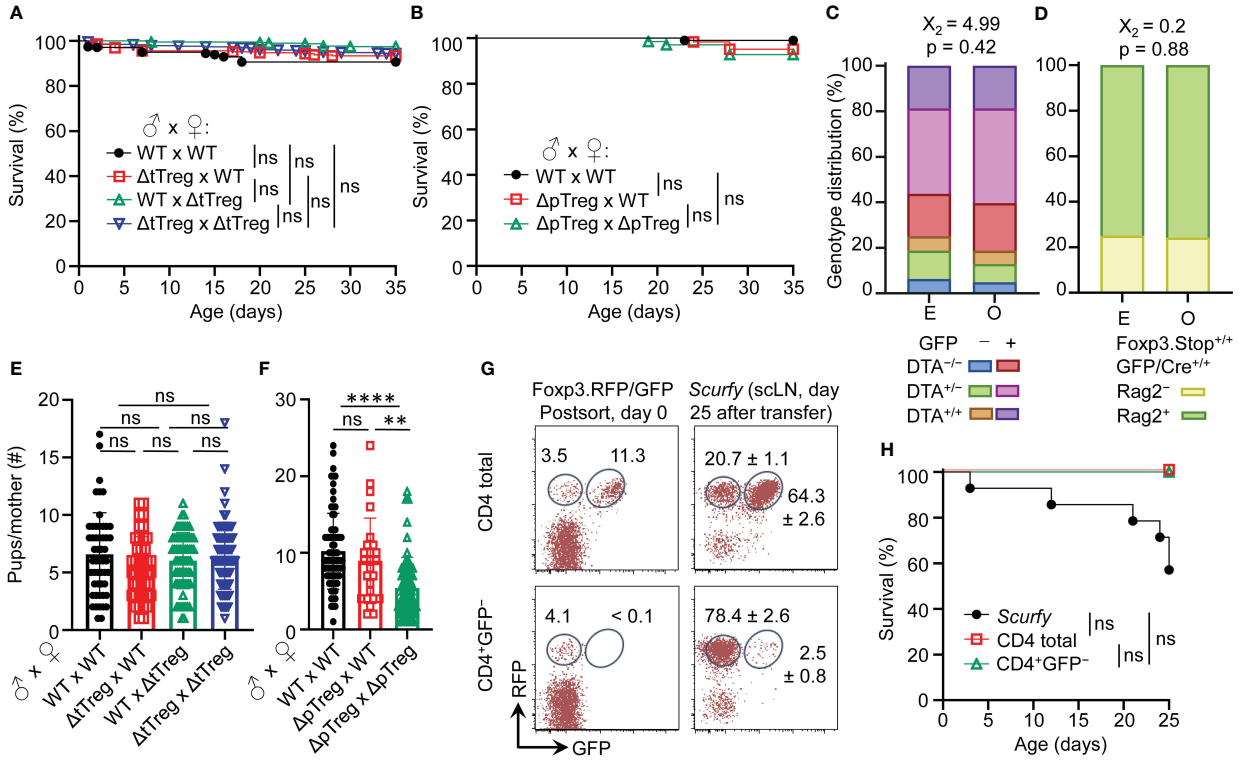

FIGURE 3
Viability and reproductive ability of ΔtTreg and ΔpTreg mice. **(A, B)** Kaplan-Meier survival curves of offspring produced by **(A)** ΔtTreg and **(B)** ΔpTreg pair mating. Newborn mice produced by indicated parental genotype combinations were monitored for the occurrence of spontaneous cases of mortality from birth onwards for up to 35 days (parental genotype, number of offspring; **(A)**: wild-type (WT) x WT, n = 342; ΔtTreg x WT, n = 227; WT x ΔtTreg, n = 398; ΔtTreg x ΔtTreg, n = 463. **(B)**: WT x WT, n = 103; ΔpTreg x WT, n = 62; ΔpTreg x ΔpTreg, n = 69). **(C, D)** Expected **(E)** and observed (O) distribution of offspring according to their genotype produced by **(C)** ΔtTreg (♂ DTA$^{+/-}$GFP$^{+/-}$ x ♀ DTA$^{+/-}$GFP$^{+/-}$; n = 187) and **(D)** ΔpTreg (♂ Rag2$^{+/-}$Foxp3.Stop^{+}GFP/Cre^{+} x ♀ Rag2$^{+/-}$Foxp3.Stop$^{+/+}$ GFP/Cre$^{+/+}$; n = 62) pair mating. Chi-square test: X_2, Chi-square. **(E, F)** The cumulative number of newborn pups produced by **(E)** ΔtTreg and **(F)** ΔpTreg pair mating of indicated parental genotype combinations. ΔtTreg: DTA$^{+/-}$ or $^{+/+}$, BAC.Foxp3$^{GFP/Cre+}$; ΔpTreg: Rag2$^{+/-}$, Foxp3.Stop$^{hemi/homo}$, BAC.Foxp3$^{GFP/Cre+}$. Parental genotype, number of litters: **(E)**: WT x WT, n = 52; ΔtTreg x WT, n = 42; WT x ΔtTreg, n = 66; ΔtTreg x ΔtTreg, n = 72. **(F)**: WT x WT, n = 75; ΔpTreg x WT, n = 25; ΔpTreg x ΔpTreg, n = 60). Symbols and bars represent individual litters and mean values, respectively. **(G, H)** Adoptive Treg cell transfer into neonatal *scurfy* mice. **(G)** Flow cytometry of RFP and GFP expression among CD4-gated T cells before (post sort, left panels) and after (day 25, right panels) injection into conventional *scurfy* recipient mice. Numbers in dot plots represent percentages of cells (left) or mean percentages of cells ± SD (right) within the respective gate. **(H)** Kaplan-Meyer survival curves of *scurfy* mice that had either been left untreated (closed black circles, n = 14) or neonatally injected *i.p.* (5 x 10^5 cells, day 0) with either total CD4$^+$ T cells (open black squares, n = 4) or CD4$^+$GFP$^-$ T cells (open green triangles, n = 3) that had been FACS-purified from peripheral lymphoid tissues of Foxp3$^{RFP/GFP}$ mice. At day 25, adoptively transferred CD4$^+$CD45.2$^+$ cells were tracked by flow cytometry in scLNs of congenic CD45.1$^+$ recipient mice and analyzed for RFP and GFP expression. **(A, B, H)** Log-rank test and Bonferroni correction: ns, not significant. **(E, F)** Unpaired t-test: ns, not significant; **p ≤ 0.01, ****p ≤ 0.0001.

RFP$^+$GFP$^-$ pTreg cells) that had been FACS-isolated from peripheral lymphoid tissues of Foxp3$^{RFP/GFP}$ mice for RFP$^+$GFP$^+$ tTreg cell depletion (Figure 3G, bottom left). In these experiments, total CD4$^+$ T cells (including RFP$^+$GFP$^-$ pTreg and RFP$^+$GFP$^+$ tTreg cells) were included for comparison (Figure 3G, top left). Both cohorts of *scurfy* recipients were viable (Figure 3H) and appeared phenotypically healthy until the end of the observation period, apart from mild symptoms of delayed growth and exfoliative dermatitis in individual mice that received tTreg cell-depleted CD4$^+$GFP$^-$ T cells (data not shown). In the absence of tTreg cells, the adoptive CD4$^+$GFP$^-$ T cell transfer resulted in a marked accumulation of RFP$^+$GFP$^-$ pTreg cells among CD4$^+$ T cells (day 0: 4.1%; day 25: 78.4 ± 2.6%) in *scurfy* recipients (Figure 3G, bottom panels), most likely due to both the proliferative expansion of preformed pTreg cells and the conversion of initially CD4$^+$Foxp3$^-$ T cells (20). In *scurfy* recipients of total CD4$^+$ T cells, RFP$^+$GFP$^-$ pTreg cell frequencies among CD4-gated cells also increased (day 0: 3.5%; day 25: 20.7 ± 1.1%), but the initial pTreg:tTreg cell ratio of 1:3 was preserved (Figure 3G, top panels). Overall, these data indicate that pTreg cells can fill up the tTreg cell niche in both ΔtTreg (Figures 1D, E) and *scurfy* mice (Figures 3G, H) while maintaining a stable RFP$^+$GFP$^-$ phenotype.

Maintenance of T cell homeostasis in adult ΔtTreg mice

Our initial characterization of young ΔtTreg mice failed to reveal evidence for disease symptoms associated with selective tTreg cell paucity. Consistently, mild immune infiltrations were limited to the salivary gland (mouse #1), and the lung (mouse #1) of individual ΔtTreg mice, but could not be observed in other organs, such as the

liver or thyroid gland (Figure 4A). Interestingly, although B6.ΔtTreg mice maintained normoglycemia, histological analyses consistently revealed pronounced immune infiltrates in the pancreas (Figure 4A, bottom panels), which is in contrast to previous studies in other settings of tTreg cell deficiency, including Foxp3-deficient mice (33) and acute Treg cell ablation in the 'Depletion of Regulatory T cells' (DEREG) mouse model on the B6 and NOD genetic background (13).

With the advancing age of ΔtTreg mice, we noticed rare cases of spontaneous deaths, which became first apparent at the age of 7 weeks ($R26^{DTA}$: 18.7%, $R26^{DTA/DTA}$: 19.0%; Figure 4B). The mortality of ΔtTreg mice further increased thereafter, reaching a plateau by 10 weeks that was maintained until the end of the 20-week observation period ($R26^{DTA}$: 25.5%, $R26^{DTA/DTA}$: 30.7%), remaining well below the high mortality of *scurfy* mice (Figure 4B). We rarely observed cases of spontaneous mortality among immunodeficient $Rag2^{-/-}$ mice, or cohorts of tTreg cell-proficient, BAC.$Foxp3^{GFP/Cre-}$ ΔtTreg littermates, ΔpTreg mice, and $Foxp3^{RFP/GFP}$ mice (Figure 4B). We further noticed that mortality appeared to be associated in part with a reduced body weight and thymic atrophy of the affected ΔtTreg mice (Figure 2B). Our subsequent analyses showed that the majority of ΔtTreg mice had a body weight corresponding to their age (85.3%), but also confirmed that individual mice failed to keep up with physiological body weight gain (Figure 4C), while the small and large intestine yielded an unsuspicious histopathological result (data not shown).

With regard to peripheral T cell homeostasis in adult ΔtTreg mice, total cellularity of scLNs (but not of mLNs and SPL; see also Supplementary Figure S3A) was moderately, although significantly increased, as compared to $Foxp3^{RFP/GFP}$ and ΔpTreg mice (Figure 5A). We only occasionally observed adult ΔtTreg mice with pronounced lymphadenopathy and splenomegaly (< 10%; Figure 5A). The analysis of inflammatory cytokine production indicated moderately increased proportions of IFN-γ- and IL-4-producing $CD4^+$ T cells (Figure 5B, top), and of IFN-γ-producing $CD8^+$ T cells (Figure 5B, bottom) in scLNs (but not in mLN or SPL; data not shown) of ΔtTreg mice. Increased expression of other cytokines (*e.g.*, IL-2, IL-10, IL-17) could also not be observed (data not shown). Consistent with largely normal frequencies of $CD4^+$ and $CD8^+$ T cells in the majority of ΔtTreg mice (Figure 1D), our flow cytometric analysis of CD62L and CD44 expression revealed no evidence for systemically uncontrolled activation of $CD4^+$ and $CD8^+$ T effector cells, neither in scLNs (Figures 5C–E) or other peripheral lymphoid tissues, such as mLN or SPL (Figures 5D, E).

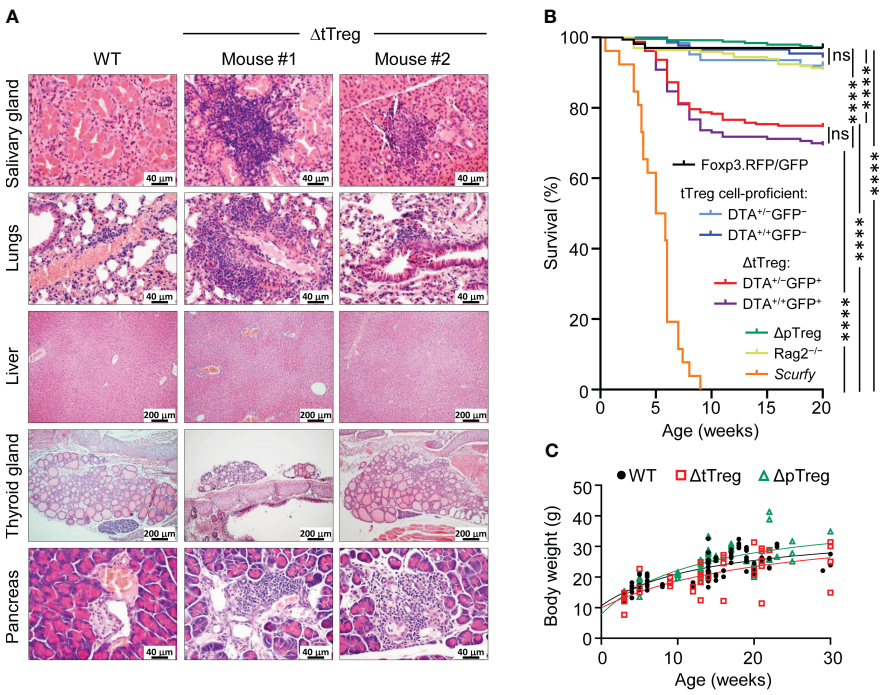

FIGURE 4
Autoimmune pathology and mortality of ΔtTreg mice. **(A)** Histological analysis. Organs of 4-week-old $Foxp3^{RFP/GFP}$ (WT, left) and ΔtTreg (mouse #1 and #2) male mice were fixed in 4% PFA, and cut sections were stained with hematoxylin and eosin. In contrast to WT controls, individual ΔtTreg mice show mild leukocytic infiltrations in the salivary gland (mouse #1: +; mouse #2: ±), lungs (mouse #1: ++; mouse #2: ±), and pancreas (mouse #1: ++; mouse #2: ++). Sections from the liver and thyroid gland of WT and ΔtTreg mice lack leukocytic infiltrates and show normal organ structure. Magnifications: Salivary gland, lung, pancreas: 400x (bar = 40 µm); liver, thyroid gland: 100x (bar = 200 µm). **(B)** Kaplan-Meier survival analysis. Cohorts of $Foxp3^{RFP/GFP}$ mice (n = 167), ΔtTreg mice ($DTA^{+/-}GFP^+$, n = 235; $DTA^{+/+}GFP^+$, n = 163), and ΔpTreg mice (n = 245) were monitored for the occurrence of spontaneous cases of mortality from birth onwards for up to 20 weeks, as indicated. $R26^{DTA}$ mice lacking the BAC.$Foxp3^{GFP/Cre}$ transgene ($DTA^{+/-}GFP^-$, n = 62; $DTA^{+/+}GFP^-$, n = 87), Foxp3-deficient *scurfy* mice (n = 26), and immunodeficient $Rag2^{-/-}$ mice (n = 196) were included for comparison. Log-rank test and Bonferroni correction: ns, not significant; ****p ≤ 0.0001. **(C)** Age-dependent body weight gain of $Foxp3^{RFP/GFP}$ (WT, closed black circles, n = 92), ΔtTreg (open red squares, n = 38), and ΔpTreg (open red triangles, n = 60) mice.

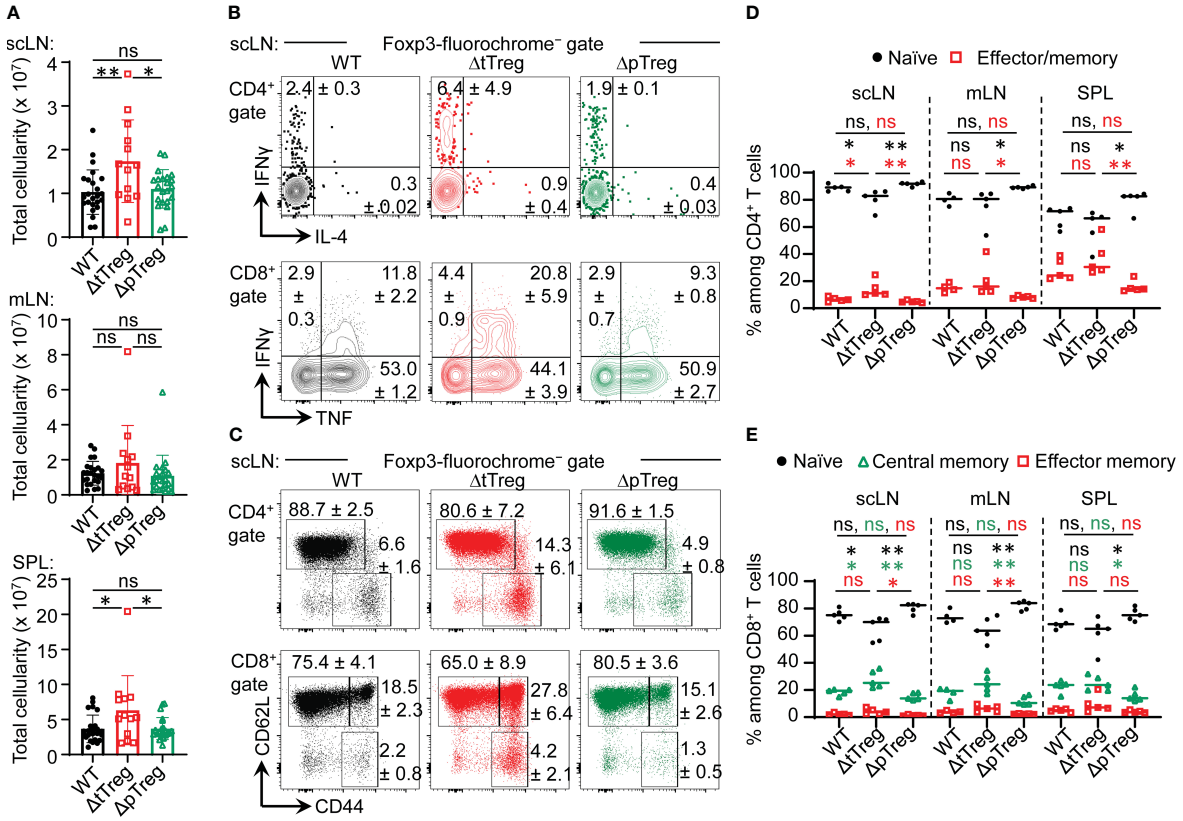

FIGURE 5
Peripheral T cell homeostasis in adult ΔtTreg and ΔpTreg mice. **(A)** Total cellularity of scLN (top), mLN, (middle), and SPL (bottom) of 13-22-week-old Foxp3$^{RFP/GFP}$ (WT, n = 24), ΔtTreg (n = 13) and, ΔpTreg (n = 22) male mice. Symbols and bars represent individual mice and mean values ± SD, respectively. **(B–E)** Flow cytometry of CD4$^+$ and CD8$^+$ T effector cells in peripheral lymphoid tissues. **(B)** Representative dot plots of IFN-γ and IL-4 expression among gated CD4$^+$Foxp3$^-$ (top), and IFN-γ and IL-4 expression among gated CD8$^+$ (bottom) T cells in scLN of 38-40-week-old WT (Foxp3$^{RFP/GFP}$), ΔtTreg, and ΔpTreg male mice, as indicated (2-3 mice per group). **(C)** Representative dot plots of CD44 and CD62L expression among gated CD4$^+$Foxp3$^-$ (top) and CD8$^+$ (bottom) T cells in scLN of 17-22-week-old WT (Foxp3$^{RFP/GFP}$), ΔtTreg, and ΔpTreg male mice, as indicated (4-5 mice per group). (Gates: CD62L^{high}CD44^{low}, naïve; CD62L^{low}CD44^{high}, effector/memory; CD62L^{high}CD44^{high}, central memory). **(D, E)** Composite percentages of T cell effector subsets in 17-22-week-old Foxp3$^{RFP/GFP}$ (WT), ΔtTreg, and ΔpTreg mice (4-5 male mice per group). **(D)** Naïve (CD62L^{high}CD44^{low}) and effector/memory (CD62L^{low}CD44^{high}) CD4$^+$ T cell compartments. **(E)** Naïve (CD62L^{high}CD44^{low}), central memory (CD62L^{high}CD44^{high}), and effector/memory (CD62L^{low}CD44^{high}) CD8$^+$ T cell compartments. Naïve: black closed circles; central memory: open green triangles; effector/memory: open red squares. Symbols and horizontal lines in **(A, D, E)** represent individual mice and mean percentages of cells ± SD, respectively. Numbers in dot plots in **(B, C)** indicate mean percentages of cells ± SD within the respective gate or quadrant. Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01.

Compensatory adaptation of pTreg cell activity in the absence of tTreg cells

Despite its already high percentage share in steady-state Foxp3$^{RFP/GFP}$ mice (32), flow cytometric immunophenotyping (Figure 6A) indicated that the percentage of pTreg cells with a CD62L^{low}CD44^{high} effector/memory-like phenotype further increased in LNs of adult ΔtTreg mice (Figure 6B), which was in contrast to tTreg cells in ΔpTreg (Figure 6C) mice. Consistent with an overall activated phenotype, pTreg cells in peripheral lymphoid tissues of ΔtTreg mice exhibited significantly upregulated expression of CD25 and several other 'Treg cell signature' proteins with functional relevance (including CD103, ICOS, ST2, and KLRG1), as compared to pTreg cells from Foxp3$^{RFP/GFP}$ mice (Figure 6D; Supplementary Figure S5, top panels). In contrast, the reduced accumulation of pTreg cells in peripheral blood of ΔtTreg mice (Supplementary Figure S2B) was accompanied by low levels of CD25 expression (Figure 6D; left panel). Additionally, tTreg cells of ΔpTreg mice exhibited neither an increased effector/memory-like compartment (Figure 6C) nor up-regulated 'Treg cell signature' protein expression (Figure 6D; Supplementary Figure S5, bottom panels), as compared to their tTreg cell counterparts in Foxp3$^{RFP/GFP}$ mice. Consistently, pTreg cells isolated from ΔtTreg mice suppressed the activity of Tresp cells more efficiently in standard cocultures, as judged by the inhibition of Tresp cell proliferation and CD25 expression, and as compared with tTreg cells from ΔpTreg mice or total Treg cells from Foxp3$^{RFP/GFP}$ mice (Figures 6E, F). In summary, in contrast to tTreg cells of ΔpTreg mice, pTreg cells in peripheral lymphoid tissues (but not blood) of ΔtTreg mice acquire a highly activated state and increased

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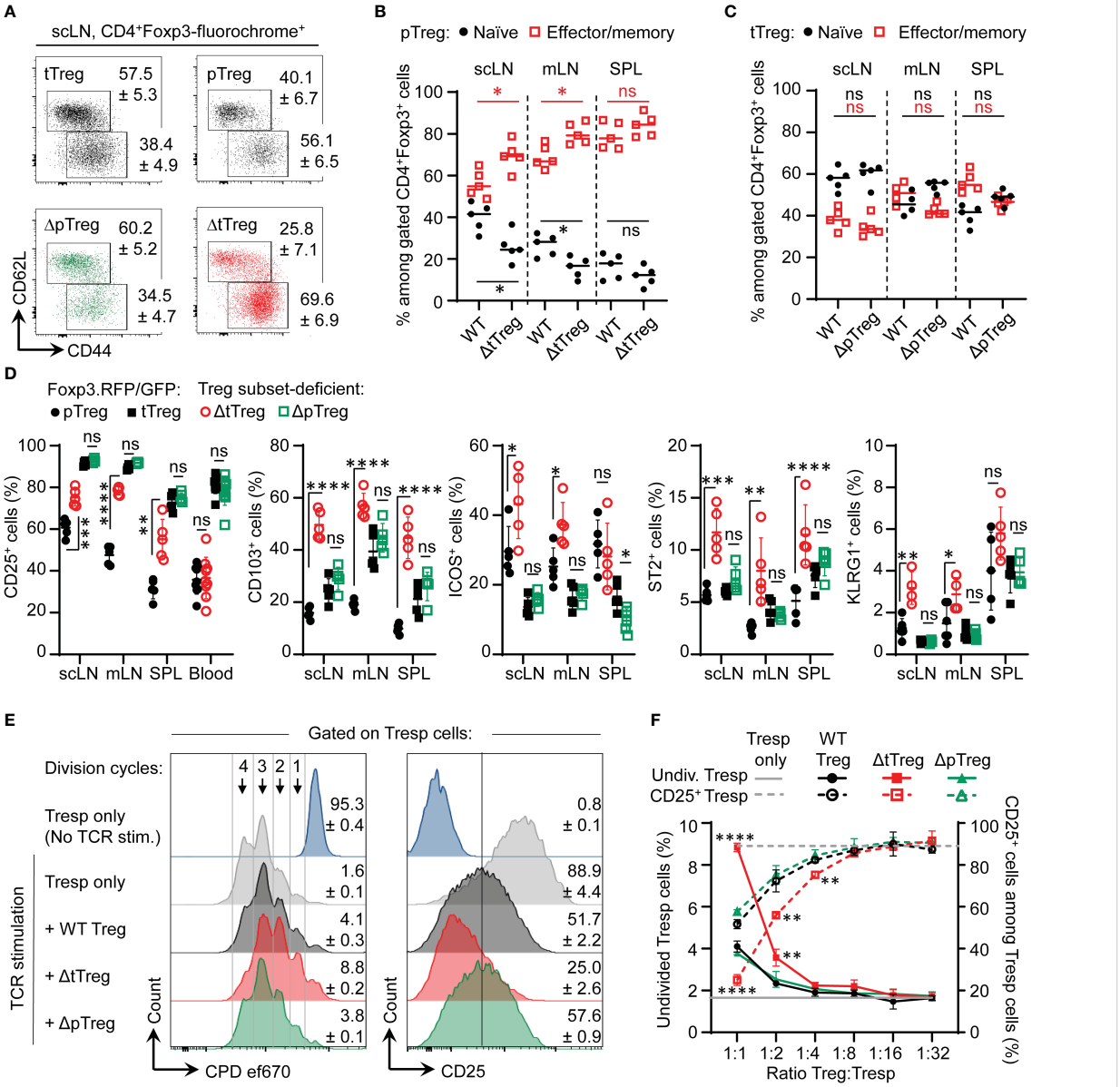

FIGURE 6
pTreg cell adaptation to tTreg cell paucity. **(A–C)** Flow cytometry of naïve and memory/effector-type Treg cell subsets. **(A)** Representative flow cytometry and cumulative percentages of **(B)** pTreg cells and **(C)** tTreg cells with a naïve (CD62L^{high}CD44^{low}, black filled circles) and effector/memory-type (CD62L^{low}CD44^{high}, red open squares) phenotype in peripheral lymphoid tissues (scLN, mLN, SPL) of ΔtTreg mice and ΔpTreg mice, respectively. Foxp3$^{RFP/GFP}$ (WT) mice were included for comparison. Treg cell gating was as in Figure 1D. **(D)** Signature protein expression. Composite percentages of surface marker expression among Foxp3-fluorochrome reporter-gated CD4$^+$ Treg cells (See Supplementary Figure S5 for representative flow cytometry). Treg cell gating was as indicated in Figure 1D: pTreg (closed black circles) and tTreg cells (closed black squares) of Foxp3$^{RFP/GFP}$ mice; pTreg cells of ΔtTreg mice (open red circles); and tTreg cells of ΔpTreg mice (open green squares). Symbols and horizontal lines indicate individual mice and mean values ± SD, respectively. Data are from a single experiment, representative of 4 independent experiments performed (n = 3-6 per group; age: 13-22 weeks). **(E, F)** Suppressor function *in vitro*. **(E)** Representative flow cytometry of T responder (Tresp) cell proliferation based on dilution of the proliferation dye (CPD) ef670 (left) and CD25 expression levels (right). Numbers in histograms indicate mean percentages of cells ± SD within the respective gate or quadrant. **(F)** Composite percentages of cell division (left, closed symbols) and CD25 expression (right, open symbols) of CD4$^+$ Tresp cells at day 3 of co-culture, using total Treg cells of Foxp3$^{RFP/GFP}$ mice (WT, black circles), pTreg cells of ΔtTreg mice (red squares), and tTreg cells (green triangles) of ΔpTreg mice at indicated Tresp : Treg ratios. For this, FACS-purified CD4$^+$CD62L$^+$Foxp3$^-$CD25$^-$ Tresp cells were co-cultured with APCs and 0.5 µg/ml α-CD3ε mAb, in the absence or presence of total Treg cells from scLN of Foxp3$^{RFP/GFP}$ mice (WT, tTreg + pTreg), pTreg cells of ΔtTreg, or tTreg cells of ΔpTreg mice. Symbols and error bars in graphs indicate mean percentages ± SD of technical replicates (n = 2-3) from one experiment, representative of three independent experiments (5-10 mice per group at 20-22 weeks of age). Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

suppressor function, which is indicative for their active involvement in constraining chronic immune dysregulation in the absence of tTreg cells (Figure 4).

Fatal autoimmune pathology in ΔtTreg mice on a mixed (B6>NOD) background

Our characterization of ΔtTreg mice on the B6 background revealed neither early- or late-onset of severe morbidity nor other signs of fatal autoimmunity (Figures 3A, 4A, B) consistently observed in mice with complete Foxp3^{+} Treg cell deficiency (2, 5). However, the severity of autoimmune pathology associated with Treg cell deficiency can be markedly shaped by genetic factors: B6 *scurfy* mice can survive for up to 9 weeks after birth (Figure 4B), which is significantly longer than *scurfy* mice on the BALB/c background, all of which succumb to death within < 5 weeks of birth (20). Here, we aimed to explore how increased genetic susceptibility impinges on the survival and immune homeostasis of ΔtTreg mice by backcrossing the B6.ΔtTreg mouse line to autoimmune-prone NOD mice carrying the dual Foxp3$^{RFP/GFP}$ reporter. On a pure NOD background, spontaneous T1D manifestation is under polygenic control of more than 20 insulin-dependent diabetes (*Idd*) gene loci (55), which additionally confer a broad susceptibility to multiple other autoimmune syndromes (peripheral neuropathy, autoimmune thyroiditis, *etc.*), albeit often with a low incidence (56). The analysis of (B6>NOD) hybrid mice obtained by two consecutive backcrosses (F2) revealed that selective tTreg cell paucity drastically decreased the survival of F2 ΔtTreg mice (both I-Ag7$^{+/-}$ and I-Ag7$^{+/+}$; see below) to ≤ 20% within 20 weeks after birth (Figure 7A), as compared to F1 ΔtTreg mice (84.2%; Figure 7A) and ΔtTreg mice on a pure B6 background (R26^{DTA}: 74.5%, R26$^{DTA/DTA}$: 69.3%; Figure 4B). Male and female F2 ΔtTreg mice showed no significant differences in mortality (data not shown). Continued backcrossing further exacerbated morbidity, such that none of the F3 ΔtTreg mice lived beyond 14 weeks (Figure 7A).

In order to account for possible variability of disease pathology on a mixed (B6>NOD) background, we produced independent cohorts of F2 and F3 ΔtTreg mice originating from unrelated (B6>NOD) backcross breedings and parental (B6>NOD)F1 mice (Figures 7B–D). When we monitored the ΔtTreg offspring for *scurfy*-like symptoms, we found that the high incidence of spontaneous mortality depicted in Figure 7A was consistently accompanied by signs of distinct, partially overlapping autoimmune diseases in independent cohorts of F2 ΔtTreg mice (Figures 7C, D). Most prominently, we observed signs of wasting disease (WD; reduced body weight and size, failure to thrive), autoimmune diabetes (hyperglycemia), and peripheral neuropathy (PN; hindlimb paralysis). In the (B6>NOD)F3 generation, ≥ 90% of ΔtTreg mice suffered from either WD (62.2%), T1D (32.4%), and/or PN (10.8%) (Figure 7B). Histopathological analyses indicated massive immune infiltrates of the salivary glands, the lung, the stomach, the thyroid glands, and the pancreas associated with severe tissue damage predominantly affecting thyroid glands and pancreatic islets of NOD.ΔtTreg F3 mice, which correlated with their hyperglycemic state (Figure 7E; top panel). A more detailed analysis of pancreatic islets (10 – 20 islets/mouse) revealed hardly any signs of immune infiltrates (mouse #1: 0%; mouse #2: < 10%; both non-diabetic) into the islets of tTreg cell-proficient (B6>NOD) F3 mice, whereas the majority of islets of ΔtTreg (B6>NOD)F3 mice (#1: > 90%, diabetic; #2: 70 – 80%; nondiabetic; #3: > 90%, diabetic) showed marked immune infiltrates (Figure 7E; data not shown). In these experiments, other organs commonly targeted by severe autoimmune responses in Foxp3-deficient mice showed no or only minimal immune infiltrates in NOD.ΔtTreg F3 mice, such as the small intestine or the liver (data not shown). Notably, severe salivary gland autoimmunity could be observed in both ΔtTreg and Foxp3$^{RFP/GFP}$ mice (Figure 7E; left panels), and thus driven by the increased genetic autoimmune risk of the (B6>NOD)F3 background, rather than selective tTreg cell paucity. Other hallmarks of the fatal autoimmune syndrome affecting Foxp3-deficient mice could not be observed, such as skin lesions or scaliness and crusting of the eyelids, ears, and tail (data not shown). Throughout the present study, the manifestation of WD, T1D, and/or PN in tTreg cell-proficient littermates of (B6>NOD) hybrid ΔtTreg mice has also not been observed (Figures 7B–E, 8A; and data not shown).

Pancreatic β cell autoimmunity in (B6>NOD) hybrid ΔtTreg mice

While Foxp3^{+} Treg cell-deficient NOD mice fail to develop insulitis and overt diabetes (33), the data depicted in Figure 7 provided the first indications that selective tTreg cell paucity can promote severe insulitis and overt diabetes (32.4%; Figures 7B–E) in both males and females, despite incomplete backcrossing onto the NOD background. However, more definite conclusions on the role of tTreg cells in controlling pancreatic β cell autoimmunity were hampered by the overall early onset of high morbidity and mortality (Figure 7A). In fact, three diabetic F3 NOD.ΔtTreg mice additionally exhibited signs of WD and PN (Figure 7B), suggesting that some (B6>NOD) hybrid ΔtTreg mice may succumb to death before the diabetes diagnosis. We therefore tracked blood glucose levels in cohorts of 3-week-old, initially normoglycemic F2 NOD.ΔtTreg mice that showed no signs of WD, PN, or other *scurfy*-like symptoms (Figure 8A). In our colony of conventional NOD mice, the first diabetes cases become apparent at approximately 12 weeks of age and continuously increase to an incidence of 70-90% in females and 0-20% in males within 30 weeks of age (13, 57). Whereas in (B6>NOD)F2 ΔtTreg mice, selective tTreg cell paucity unleashed a particularly severe form of T1D: > 50% of males rapidly progressed to overt diabetes within < 8 weeks after birth (Figure 8A), despite the usually observed female sex bias and kinetics difference in the NOD model (58). Flow cytometry-based MHC class II haplotyping indicated that diabetes manifestation in F2 ΔtTreg mice correlated with homozygous expression of the diabetogenic MHC class II molecule I-Ag7 of the NOD genetic background (*Idd1*), whereas

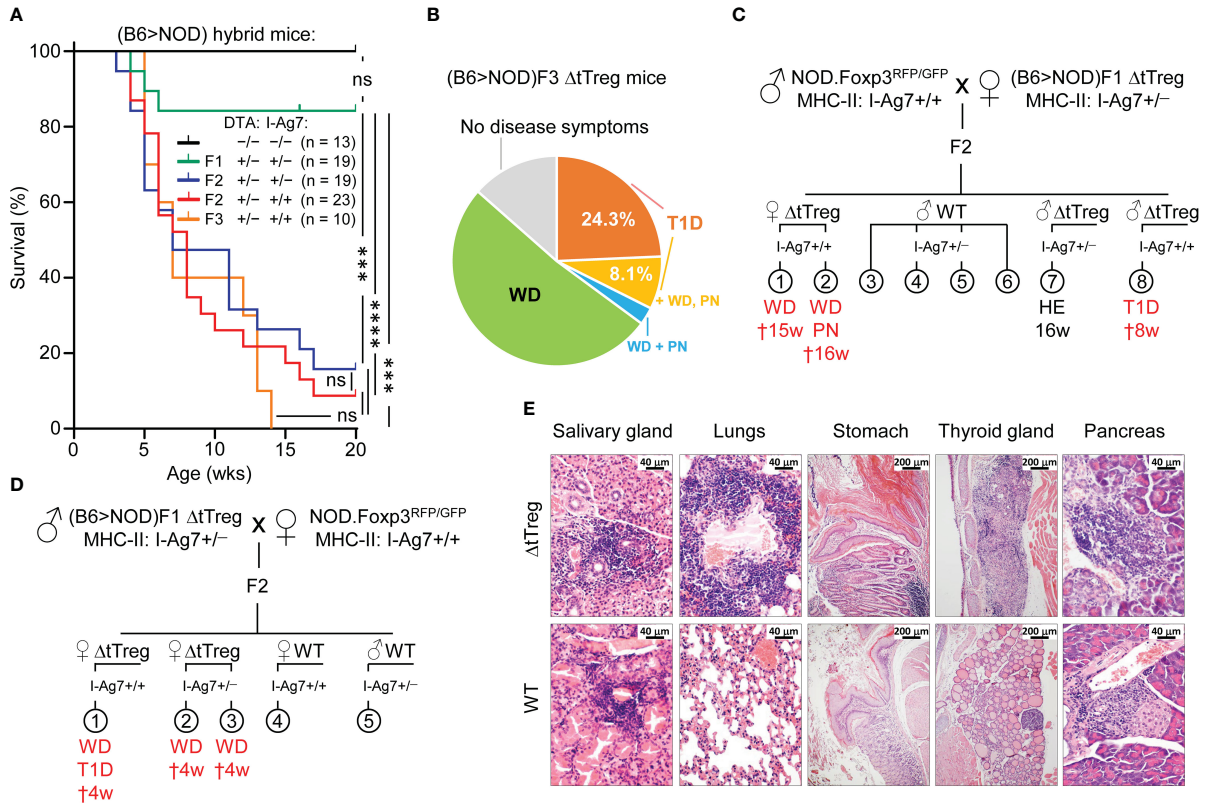

FIGURE 7
Spontaneous mortality and autoimmune pathology in ΔtTreg mice on a mixed (B6>NOD) background. B6.ΔtTreg mice were backcrossed to NOD.Foxp3$^{RFP/GFP}$ mice for up to 3 generations, as indicated (F1, F2, F3). (B6>NOD) hybrid offspring were analyzed by flow cytometry for the haplotype-specific expression of MHC class II molecules (NOD: I-A^{g7}; B6: I-A^{b}). **(A)** Kaplan-Meier survival analysis. Cohorts of DTA$^{+/-}$ ΔtTreg mice on a mixed (B6>NOD) background and with heterozygous I-Ag7$^{+/-}$ (F1, n = 19; F2, n = 19) or homozygous I-Ag7$^{+/+}$ (F2, n = 23; F3, n = 10) expression were monitored for the occurrence of spontaneous cases of mortality and morbidity from birth onwards for up to 20 weeks, as indicated. Note that tTreg cell-proficient B6.Foxp3$^{RFP/GFP}$ mice (DTA$^{-/-}$, I-Ag7$^{-/-}$; n = 13) were included for comparison. Log-rank test and Bonferroni correction: ns, not significant; ***p ≤ 0.001, ****p ≤ 0.0001. **(B)** Morbidity of (B6>NOD)F3 ΔtTreg mice (37 males and females from 7 litters). WD: 51.4%, T1D: 24.3%, T1D + WD + PN: 8.1%, WD + PN: 2.7%, no disease symptoms: 13.5%. **(C, D)** Representative pedigrees of **(C)** (NOD>F1) and **(D)** (F1>NOD) ♂ x ♀ matings and health status of resultant F2 offspring. Parental (B6>NOD)F1 mice in **(B, C)** were obtained from independent (B6>NOD) backcross breedings. WD, wasting disease; T1D, type 1 diabetes; PN, peripheral neuropathy; HE, healthy (no disease symptoms); †: age of death in weeks. **(E)** Histological analysis. Organs of 11-13-week-old (B6>NOD)F3 males (I-Ag7$^{+/+}$) were fixed in 4% PFA and cut sections were stained with hematoxylin and eosin. Representative histology showing pronounced leukocytic infiltrations and severe histopathological changes (score: +++) in the salivary gland, lungs, thyroid gland, stomach, and pancreas of a hyperglycemic R26^{DTA} NOD.ΔtTreg F3 mouse (top panels). R26$^{wt/wt}$ NOD.Foxp3$^{RFP/GFP}$ F3 mice (WT, bottom panels) show comparably severe immune infiltrates in the salivary glands, but only marginal (lungs, pancreas) or no (stomach, thyroid gland) autoimmune infiltration in other organs. Magnifications: Salivary gland, lung, pancreas: 400x (bar = 40 µm), stomach, thyroid gland: 100x (bar = 200 µm).

mice co-expressing I-Ab of B6 origin remained normoglycemic during the observation period (Figure 8A). In contrast, high mortality (Figure 7A) and the manifestation of WD (Figure 7D) was independent of homozygous I-Ag7 expression.

In line with the absence of severe systemic autoimmune responses, pLNs of F2 ΔtTreg mice showed clear signs of lymphadenopathy, whereas the size of non-draining LNs (scLNs, mLNs) and SPL did not significantly differ between tTreg cell-deficient and -proficient (B6>NOD)F2 mice (Figure 8B). Consistently, numbers of CD8$^+$ and CD4$^+$ T cells (Supplementary Figure S6A, top panels) were selectively increased in pLNs of F2 ΔtTreg mice.

Consistent with our data in B6.ΔtTreg mice (Figures 1D, E), efficient intrathymic tTreg cell ablation in (B6>NOD)F2 ΔtTreg mice (Supplementary Figure S6B) was accompanied by a significant, up to 5.5-fold increase in the percentage of RFP$^+$GFP$^-$ pTreg cells among CD4-gated T cells in peripheral lymphoid tissues (Figures 8C, D; see Supplementary Figure S6A for Treg cell numbers). However, in contrast to B6.ΔtTreg mice (Supplementary Figure S2A), the population size of pTreg cells in (B6>NOD)F2 ΔtTreg mice only partially compensated for the numerical impairment of the overall Treg cell pool in the absence of tTreg cells (Figure 8D; Supplementary Figure S6A, bottom). Additionally, thymic cellularity (Supplementary Figure S6C) and numbers of T cell developmental stages (Supplementary Figure S6D) were consistently reduced in (B6>NOD)F2 ΔtTreg mice, as compared to in (B6>NOD)F2 Foxp3$^{RFP/GFP}$ mice, probably due to increased hyperglycemia-induced stress and/or systemically elevated inflammatory cytokine levels.

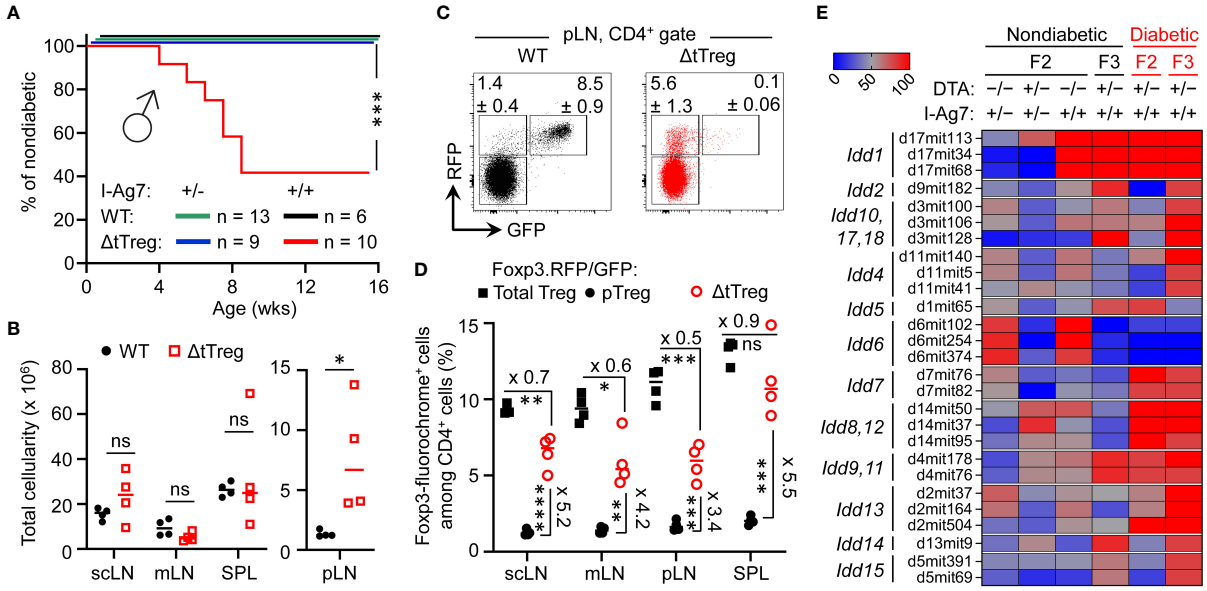

FIGURE 8
Pancreatic β cell autoimmunity in hybrid (B6>NOD) ΔtTreg mice. **(A)** Blood glucose levels of 3-week-old F2 ΔtTreg males with heterozygous (I-Ag7$^{+/-}$, n = 9) or homozygous (I-Ag7$^{+/+}$, n = 10) expression of I-Ag7 were monitored once a week. I-Ag7$^{+/-}$ (n = 13) and I-Ag7$^{+/+}$ (n = 6) F2 Foxp3$^{RFP/GFP}$ mice (WT) mice were included for comparison. Note that initially normoglycemic mouse cohorts were selected based on the absence of WD, PN, or other *scurfy*-like symptoms. Log-rank test: ns, not significant; ***p ≤ 0.001. **(B–D)** Flow cytometric immunophenotyping of (B6>NOD)F2 mice depicted in Figure 8A. **(B)** Total cellularity of pLN (right) and other peripheral lymphoid tissues (scLN, mLN, SPL) of Foxp3$^{RFP/GFP}$ (closed black circles) and ΔtTreg (open red squares) mice (all 8-week-old I-Ag7$^{+/+}$ males). See Supplementary Figure S6A for corresponding numbers of CD8$^+$ and CD4$^+$ T cells. **(C)** Representative dot plots of Foxp3-driven RFP and GFP expression among CD4-gated cells from pLN from Foxp3$^{RFP/GFP}$ and ΔtTreg mice, and **(D)** cumulative percentages of total Treg (closed black squares) and pTreg (closed black circles) cells of Foxp3$^{RFP/GFP}$ mice, as well as pTreg cells of ΔtTreg mice (open red circles) from peripheral lymphoid tissues, as indicated (all I-Ag7$^{+/+}$). Symbols and horizontal lines in **(B, D)** indicate individual mice and mean values of 4 mice per group, as depicted in Figure 8A. Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. **(E)** Genomic DNA-based *Idd* gene locus analysis in cohorts of (B6>NOD) hybrid mice. Cohorts of F2 Foxp3$^{RFP/GFP}$ mice (WT; I-Ag7$^{+/-}$: n = 17; I-Ag7$^{+/+}$, n = 11); F2 ΔtTreg mice (I-Ag7$^{+/-}$, n = 7; I-Ag7$^{+/+}$, n = 5), and F3 ΔtTreg mice (nondiabetic I-Ag7$^{+/+}$, n = 8; diabetic I-Ag7$^{+/+}$, n = 5) were subjected to genomic PCR for *Idd* gene analysis. The heatmap shows the distribution of a selected set of *Idd* loci among different experimental groups, as indicated. For this, the percentage of mice homozygous for the respective NOD *Idd* gene locus was calculated and expressed as color code. An overview of the complete data set is provided in Supplementary Figure S7. Note that the R26-DTA transgene of ΔtTreg mice is embedded within the *Idd6* gene locus, resulting in a marked underrepresentation of *Idd6* in F2 and F3 ΔtTreg mice.

Contribution of NOD *Idd* loci to diabetes in (B6>NOD) hybrid ΔtTreg mice

We found that the manifestation of overt diabetes was restricted to I-Ag7$^{+/+}$ (B6>NOD) hybrid ΔtTreg mice (Figures 7B–E, 8A), consistent with the requirement of *Idd1* homozygosity for high penetrance of diabetes susceptibility in the NOD model (59). In fact, *Idd1* was shown to confer most of the diabetes risk (60), but not to be sufficient to precipitate diabetes in Foxp3$^+$ Treg cell-proficient NOD mice (60). We hypothesized that the early manifestation of diabetes in I-Ag7$^{+/+}$ F2 ΔtTreg males with high penetrance (Figure 8A) was driven by the acquisition of one or more additional, non-MHC-linked *Idd* loci. As expected, after only two backcross generations, PCR-based genomic *Idd* gene analysis (Supplementary Figure S7) indicated that the majority of *Idd* genes included in our survey was dispersible for diabetes development in I-Ag7$^{+/+}$ F2 ΔtTreg mice (*Idd2*, *Idd3/Idd10/Idd17/Idd18*, *Idd4*, *Idd14*, *Idd15*) (Supplementary Figure S7A). This included *Il2* gene polymorphisms (encoded by *Idd3*), which play an important role in the reduced IL-2 receptor signaling strength received by Treg cells in conventional, tTreg cell-proficient NOD mice, resulting in their functional deficiency (61, 62). Additionally, *Idd6* was markedly underrepresented in (B6>NOD) F2 (Supplementary Figure S7A; Figure 8E) and F3 (Supplementary Figure S7B; Figure 8E) ΔtTreg mice, as compared to their Foxp3$^{RFP/GFP}$ littermates, which can be attributed to the genomic localization of the R26-DTA transgene of ΔtTreg mice within the *Idd6* gene locus (http://www.informatics.jax.org/). Other *Idd* gene loci initially underrepresented in diabetic F2 ΔtTreg mice were found to be enriched after continued backcrossing (*Idd2, Idd4, Idd3/Idd10/Idd17/Idd18, Idd13.1/.2, Idd14, Idd15)* (Supplementary Figure S7B; Figure 8E).

In addition to *Idd1*, a set of 5 *Idd* gene loci (*Idd5.1*, *Idd7*, *Idd8/Idd12*, *Idd9.1/.2*, *Idd13.3*) was detectable in ≥ 80% of diabetic F2 ΔtTreg mice (Figure 8E), but was not sufficient to promote diabetes in tTreg cell-proficient I-Ag7$^{+/+}$ (B6>NOD)F2 littermates (Figure 8A; and data not shown). Interestingly, this rather small set of *Idd* loci was primarily characterized by harboring genes with well-known functions in the development, survival/maintenance, function of pancreatic β cells [*Idd7*, *Idd8*, *Idd12* (63–67)] and Foxp3$^+$ Treg cells (*Idd5*, *Idd7*, *Idd9.1/.2*, *Idd13.3*). In particular, several annotated genes located in *Idd5* [*Cd28*, *Icos*, *Ctla4* (68, 69); *Pdcd1* (70); *Irs1*, *Stat1* (71); *Ikfz2* (72)] and the *Idd9* gene locus [(*Cd30, Tnfr2, Cd137* (73, 74); *p110δ, mTOR* (75, 76)] play key roles

in various facets of Foxp3$^+$ Treg cell biology (77–81), many of which belong to the shared transcriptional signatures of tissue-type Treg (22, 82, 83) and pTreg (32) cells. Furthermore, and directly relevant to the autoimmune mechanisms underlying pancreatic β cell destruction, *Idd7* (containing *Nfkbid*) has been implicated in modulating diabetogenic CD8$^+$ T cell deletion in the thymus (84) and numbers and suppressor function of Foxp3$^+$ Treg cells in peripheral tissues (85). Lastly, several annotated genes encoded in *Idd13* [*B2m* (86, 87); *Mertk* (88); *Bcl2l11/Bim, Id1, Smox, Pdia3* (89)] play key roles in development and activation of diabetogenic T effector cells (86, 87), including negative thymic selection (88–90).

Overall, these findings in ΔtTreg mice are consistent with a scenario, in which pTreg cell-mediated maintenance of immunological tolerance to pancreatic β cells can be abrogated by the acquisition of a limited set of *Idd* risk loci, some of which unfold their diabetogenic activity directly in pTreg cells. In support of this interpretation, comparative flow cytometry-based immunophenotyping revealed a correlation of some detected *Idd* loci and differential protein expression in pTreg cells of F2 ΔtTreg mice, as compared to pTreg cells in tTreg cell-proficient Foxp3$^{RFP/GFP}$ littermates (Figure 9). This included the absence of *Idd3* (including *Il2*) and markedly increased expression levels of CD25 on pTreg cells from pLNs of diabetic F2 ΔtTreg mice, as compared to F2 Foxp3$^{RFP/GFP}$ (Figures 9A, C; left panels). Relevant to their high diabetes susceptibility, the acquisition of *Idd5.1* (68) by F2 ΔtTreg mice correlated with increased expression levels of ICOS (*Icos*) and PD-1 (*Pdcd1*) on pTreg cells (Figures 9A, C), and the accumulation of an unusual ICOS$^+$PD-1^{high} pTreg cell subset in F2 ΔtTreg mice, but not in Foxp3$^{RFP/GFP}$ littermate controls (Figure 9D). In F2 ΔtTreg mice, other Treg signature proteins were either expressed on a higher proportion of pTreg cells (*e.g.*, Nrp1, CD103, KLRG1) or were expressed at higher levels (GITR) (Figure 9B). Importantly, functional incapacitation of PD-1 in gene-targeted mice (91–93) and human patients treated with blocking Abs (94) can result in overt autoimmune responses, including T1D (94–98), indicating a primarily inhibitory function of PD-1 expression in immune effector cells. However, independent lines of evidence in mice have pointed towards a diabetogenic role of PD-

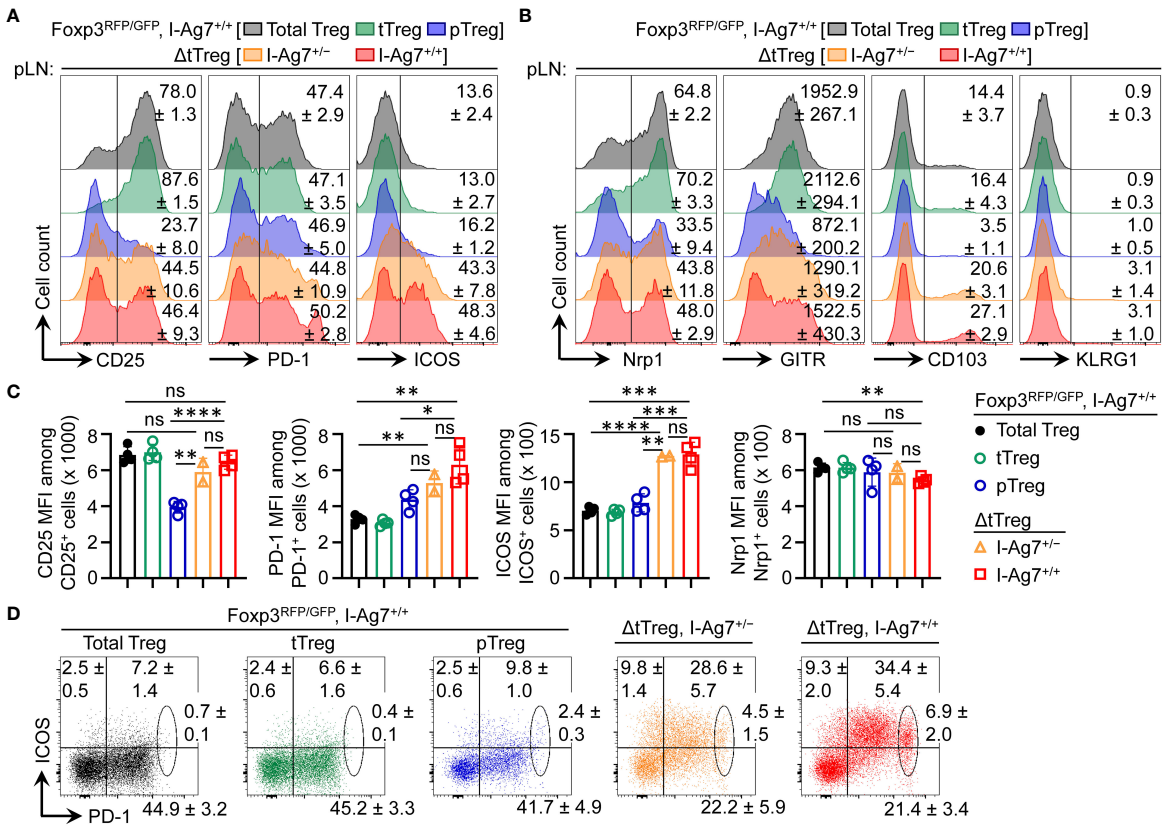

FIGURE 9
Flow cytometry-based immunophenotyping of pTreg cells in (B6>NOD)F2 ΔtTreg mice. Expression analysis of selected Treg cell signature proteins on gated Treg cell subsets of I-Ag7$^{+/+}$ Foxp3$^{RFP/GFP}$ mice (total Treg: grey; tTreg: green; pTreg: blue) and pTreg cells of ΔtTreg mice that were either I-Ag7$^{+/-}$ (orange) or I-Ag7$^{+/+}$ (red). Surface expression of **(A)** CD25, PD-1, and ICOS, and of **(B)** Nrp1, GITR, CD103, and KLRG1 on pTreg cells from F2 ΔtTreg mice, as compared to pTreg cells from F2 Foxp3$^{RFP/GFP}$ mice. Numbers in histograms indicate mean percentages of cells ± SD within the respective gate or quadrant, with the exception of GITR in **(B)** showing mean fluorescence intensity (MFI) of the fluorochrome-conjugated mAb **(C)** Quantification of indicated marker expression based on MFI of fluorochrome-conjugated mAbs. Symbols and bars represent individual mice and mean values ± SD, respectively. **(D)** Expression of ICOS and PD-1 on pTreg cells from pLNs of Foxp3$^{RFP/GFP}$ mice and ΔtTreg mice, as indicated. Numbers in dot plots indicate mean percentages of cells ± SD within the respective quadrant or gate. Note that gated populations of total Treg cells and tTreg cells from Foxp3$^{RFP/GFP}$ mice were included for comparison. Data are from a single experiment (n = 4). Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

1 expression on Foxp3$^+$ Treg cells, which included diabetes amelioration in congenic B10.Idd5$^+$ and Idd5.1$^+$ NOD mice (69, 99), diabetes protection of NOD mice with Foxp3$^+$ Treg cell-specific PD-1 deletion (100), and an inverse correlation of PD-1 expression with the expression of Foxp3 and Foxp3$^+$ Treg cell function (100, 101). Clearly, future studies are warranted to further dissect the differential function of PD-1 on T effector and Foxp3$^+$ Treg cells, including pTreg cells.

Discussion

As the functional heterogeneity of pTreg and tTreg cells promises to enable the subset-specific therapeutic manipulation of their activity in various clinical settings, it will be important to define their exact roles in establishing and maintaining peripheral immune homeostasis. The selective ablation of the development of pTreg cells (26, 27, 102) and tTreg cells, as done here, represents a considerable improvement over previous experiments relying on Foxp3-deficient mice and their reconstitution by adoptive Treg cell transfer, allowing the *in vivo* consequences to be analyzed under near-physiological conditions, including minimal autoimmune perturbations. In fact, some of the pathology observed in Foxp3-deficient mice has been attributed to the enhanced thymic export and peripheral accumulation of Treg cell-like 'wanna-be' CD4$^+$ T cells with self-reactive specificities and distinct pathological properties (9, 10), rather than the mere absence of a functional Foxp3$^+$ Treg cell pool. Additionally, some defects of Foxp3-deficient mice (*e.g.*, defective lympho-hematopoiesis) are refractory to adoptive Treg cell therapy, even when total CD4$^+$ T cell populations were used (50). Here we have analyzed how selective tTreg cell paucity, which was achieved by intrathymic tTreg cell ablation while preserving pTreg cell generation, impinges on peripheral immune homeostasis in non-autoimmune and autoimmune-prone mice. Our data in B6.ΔtTreg mice reveal the ability of pTreg cells to establish immune homeostasis after birth, maintain immune tolerance in young mice, and constrain catastrophic autoimmune responses during aging in the majority of B6.ΔtTreg mice. Consistently, neonatal transfer of total CD4$^+$ T cell populations, which had been depleted of tTreg cells, ameliorated clinical signs of Foxp3 deficiency in *scurfy* recipient mice. The manifestation of some mild disease symptoms (moderate growth delay and mild exfoliative dermatitis) can probably be attributed to an initial lag phase after tTreg cell-depleted CD4$^+$ T cell transfer, which is required for seeding and proliferative expansion of pre-formed pTreg cells, and the lymphopenia-driven *de novo* generation of Foxp3$^+$ pTreg cells (20).

Besides the absence of *scurfy*-like symptoms, several additional observations in the B6.ΔtTreg model further support our interpretation that physiologic pTreg cell populations can efficiently constrain autoimmune responses in the absence of tTreg cells. This includes an overall normal size of peripheral lymphoid tissues and T effector cell compartments (numbers, activation state, inflammatory cytokine production, etc.), as well as unperturbed lympho-hematopoiesis, representing a particularly sensitive indicator for the absence of ongoing (auto)immune responses. Many organs of B6.ΔtTreg mice, which are commonly targeted by severe autoimmune destruction in Foxp3-deficient mice, show no or only mild immune infiltrations not accompanied by any appreciable tissue destruction. Thus, the underlying cause promoting the occurrence of spontaneous deaths from an age of > 7 weeks onwards has remained less clear but may involve the exacerbation of chronic, low-level inflammation in individual organs, such as the thyroid gland or the lungs (Figure 4A), rather than multi-organ autoimmunity observed in Foxp3-deficient models of complete Treg cell deficiency. Interestingly, extending our histopathological analyses of the lungs (Figure 4A) to the upper respiratory tract of ΔtTreg mice that presented with reduced body weight provided first evidence for unexpected, severe inflammatory changes in the area of the nasal and oral cavities, pointing towards decreased food intake as a possible reason underlying a reduced body weight and morbidity of this particular disease subphenotype (data not shown).

Considering the age-dependent increase in spontaneous deaths (Figure 4B), the abrogation of immune homeostasis in individual ΔtTreg mice is likely to involve immunological and/or environmental cues (102–104), which are subject to age-related changes. This may include differences in the exposure to antigens derived from the diet and commensal microbiota promoting the physiologic induction of pTreg cells (103, 105–107). Our efforts to further analyze the immune events associated with the age-related impairment of peripheral immune homeostasis in ΔtTreg mice have been hampered by the relatively low incidence of mortality, in conjunction with rapid disease progression. In-depth flow cytometry-based immunophenotyping failed to reproducibly reveal age-related changes in the peripheral immune effector compartments of ΔtTreg mice, including CD4$^+$ and CD8$^+$ T effector compartments (Figures 5C–E). While this could be taken as an indication for quantitative and/or qualitative changes affecting the pTreg cell compartment, our analyses have not provided any evidence for an age-related reduction of pTreg cell numbers or phenotypic changes in the peripheral pTreg pool. In contrast, we found that the increased pTreg cell population size largely compensated for the numerical impairment of the overall Treg cell pool in adult ΔtTreg mice (Figures 1D, E; Supplementary Figures S1B, S2), which also holds true for ΔtTreg mice that were affected by reduced body weight and thymic atrophy (Figure 2B).

At present, we can only speculate on whether the pTreg cell niche in peripheral lymphoid tissues of ΔtTreg mice is replenished early in life and then maintained by proliferative expansion of pre-formed Treg cells, or whether continuous incorporation of newly formed cells is required to maintain a peripheral pTreg cell pool and immune homeostasis. This raises the possibility that the observed age-dependent increase in spontaneous mortality and morbidity is, at least in part, associated with a reduced efficiency in pTreg cell generation. In fact, pTreg cells are thought to be mainly, if not exclusively drawn from initially naïve CD4$^+$ T cells (108). However, rates of thymic export of newly formed CD4$^+$ T cells to peripheral sites

of pTreg cell generation continuously decrease during aging and involution of the thymus (109, 110), but also during thymic atrophy due to chronic inflammatory stress (Figure 2B; Supplementary Figure S6C). Consistently, immediate CD25^{high}Foxp3^{-} pTreg cell precursors residing in peripheral lymphoid tissues of nonmanipulated mice are strongly enriched among recent thymic emigrants (18).

Overall, our findings are consistent with a scenario, in which pTreg cells in peripheral lymphoid tissues of B6.ΔtTreg mice acquire a highly activated phenotype and increased suppressor function to cope with latent, chronic autoimmune responses due to the absence of tTreg cells. This intricate equilibrium can get out of control even by subtle age-related immunological and/or microenvironmental changes, which then tip the balance in favor of fatal autoimmunity. This may include changes in the commensal microbiota and qualitative differences among the pTreg cell pool, *e.g.*, reduced rates of pTreg cell *de novo* generation, in conjunction with proliferative pTreg cell expansion narrowing the TCR repertoire.

This interpretation was further corroborated by the dramatically increased mortality associated with the early onset of severe autoimmune diseases that could be observed in ΔtTreg mice after only two backcross generations onto the autoimmune-prone NOD background. Here we focused our analysis on pancreatic β cell autoimmunity, as T1D is considered a paradigmatic autoimmune disease for the application of Treg cell-based therapies to prevent or interfere with ongoing autoimmune destruction, although the main regulator(s) of pancreatic β cell autoimmunity hasn't been identified yet. Lastly, Foxp3-deficient NOD mice with a polyclonal CD4^{+} T cell repertoire fail to present with insulitis and overt diabetes before they succumb at 3 weeks to severe inflammatory infiltration in multiple organs (33), precluding NOD.Foxp3-deficient mice as an experimental model to study the role of Treg cells in the autoimmune β cell protection. Our data show that the acquisition of a small set of *Idd* risk loci, many of which encode genes with well-known functions in Treg cell biology, is sufficient to precipitate a particularly severe form of autoimmune diabetes in ΔtTreg mice on a mixed (B6>NOD) background. In this context, it is of interest to note that our complementary studies in ΔpTreg mice on a (B6>NOD)F5 background haven't provided evidence for a protective role of pTreg cells in the control of β cell autoimmunity (D.M.Z. and K.K., unpublished observation). Our observations in (B6>NOD) hybrid mice with selective tTreg cell paucity, in conjunction with previous experiments in Foxp3.CNS1$^{-/-}$ with impaired pTreg cell development (34) indicate that tTreg cells are key regulators of β cell autoimmunity in the NOD model. Clearly, future experiments are warranted using Treg cell-subset-deficient mice on a pure NOD background to provide a more definite answer on the role of tTreg and pTreg cells in the control of β cell autoimmunity.

In conclusion, ΔtTreg and ΔpTreg mice offer to directly analyze the individual roles of tTreg and pTreg cells, respectively, in the control of immune homeostasis and organ-specific autoimmunity under near-physiologic conditions, which will facilitate future studies on the functional heterogeneity of the mature Treg cell pool. Besides autoimmune diseases, of particular interest will be to dissect their subset-specific contributions to non-immune functions that have recently been attributed to tissue-type Treg cells, which include facilitating homeostasis and regeneration of nonlymphoid tissues.

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 approved by Landesdirektion Dresden, Germany. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AY: Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization. DZ: Writing – review & editing, Formal analysis, Investigation. RM: Writing – review & editing, Formal analysis, Investigation. CR: Writing – review & editing, Formal analysis, Investigation. PU: Writing – review & editing, Formal analysis, Investigation. EK: Writing – review & editing, Formal analysis, Investigation. MB: Writing – review & editing, Formal analysis, Investigation, Methodology, Project administration. DV: Writing – review & editing, Conceptualization, Formal analysis, Resources. OK: Writing – review & editing, Formal analysis, Investigation, Methodology, Visualization. SS: Writing – original draft, Conceptualization, Formal analysis, Methodology, Resources, Supervision. KK: Writing – original draft, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported with funds from the Technische Universität Dresden (TUD), Center for Molecular and Cellular Bioengineering (CMCB), Center for Regenerative Therapies Dresden (CRTD); from the German Ministry of Education and Research to the German Center for Diabetes Research (DZD e.V.); from the European Commission and EUREKA Eurostars-3 joint program (siaDM, E! 1856), and from the DFG (German Research Foundation) (FOR2599) to KK. Additionally, AY received financial support from the Graduate Academy, supported by Federal and State Funds.

Acknowledgments

The authors would like to thank Sonja Schallenberg (CRTD) for helpful discussions and constructive advice; Lena Biedermann (CRTD) for technical assistance; and Katja Bernhardt and Anne Gompf (CMCB Flow Cytometry Core Facility) for expert help in flow cytometry and cell sorting. Additionally, the authors are greatly

indebted to Abdel Gdoura and Peter van Endert (Université Paris Cité, INSERM) for sharing their longstanding expertise in *Idd* gene analysis. We also would like to thank Basak Torun for critically reading of our manuscript. A preprint of the manuscript has been deposited in bioRxiv (111).

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1298938/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 12 January 2024
DOI 10.3389/fimmu.2023.1321228

OPEN ACCESS

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RECEIVED 13 October 2023
ACCEPTED 19 December 2023
PUBLISHED 12 January 2024

CITATION
Santosh Nirmala S, Kayani K, Gliwiński M, Hu Y, Iwaszkiewicz-Grześ D, Piotrowska-Mieczkowska M, Sakowska J, Tomaszewicz M, Marín Morales JM, Lakshmi K, Marek-Trzonkowska NM, Trzonkowski P, Oo YH and Fuchs A (2024) Beyond FOXP3: a 20-year journey unravelling human regulatory T-cell heterogeneity. *Front. Immunol.* 14:1321228. doi: 10.3389/fimmu.2023.1321228

Beyond FOXP3: a 20-year journey unravelling human regulatory T-cell heterogeneity

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The initial idea of a distinct group of T-cells responsible for suppressing immune responses was first postulated half a century ago. However, it is only in the last three decades that we have identified what we now term regulatory T-cells (Tregs), and subsequently elucidated and crystallized our understanding of them. Human Tregs have emerged as essential to immune tolerance and the prevention of autoimmune diseases and are typically contemporaneously characterized by their $CD3^+CD4^+CD25^{high}$ $CD127^{low}FOXP3^+$ phenotype. It is important to note that $FOXP3^+$ Tregs exhibit substantial diversity in their origin, phenotypic characteristics, and function. Identifying reliable markers is crucial to the accurate identification, quantification, and assessment of Tregs in health and disease, as well as the enrichment and expansion of viable cells for adoptive cell therapy. In our comprehensive review, we address the contributions of various markers identified in the last two decades since the master transcriptional factor FOXP3 was identified in establishing and enriching purity, lineage stability, tissue homing and suppressive proficiency in $CD4^+$ Tregs. Additionally, our review delves into recent breakthroughs in innovative Treg-based therapies, underscoring the significance of distinct markers in their therapeutic utilization. Understanding Treg subsets holds the key to effectively harnessing human Tregs for immunotherapeutic approaches.

KEYWORDS

regulatory T cells, FOXP3, Treg markers, Treg heterogeneity, Treg function, Treg therapy, Treg chemokine receptors

1 Introduction

FOXP3$^+$ regulatory T-cells (Tregs) represent 4-7% of the CD4$^+$ T-cell population and are essential for regulating peripheral tolerance and immune homeostasis. Early phase clinical trials have demonstrated proof-of-principle for the use of isolated and expanded polyclonal Tregs in the treatment of inflammatory disorders, transplant rejection and autoimmune diseases. Treg research traces its origins back more than 40 years. The first evidence for thymus-derived cells with suppressive function came from mouse thymectomy experiments conducted in the early 1970s (1). Mice thymectomised between day two and four of life developed severe autoimmunity, which did not occur if thymectomy was performed before 24 hours or after day five of life (2). This led to the hypothesis that autoreactive T-cells are exported from the thymus within the first few days of life, followed later by an anergising subset of T-cells. Importantly, autoimmunity could be reversed with thymic transplantation at day ten. In line with these findings, Gershon and Kondo showed thymus-derived lymphocytes were crucial for inducing tolerance (3), and tolerance could be adoptively transferred to naïve recipients (4). The presence of suppressor T-cells, however, was questioned when several other groups failed to verify the mechanisms of suppression postulated using the novel techniques, including monoclonal antibodies and Sanger sequencing. These negative findings together with a lack of specific markers led to a loss of interest in the suppressor T-cell theory in the mid-1980s.

In the 1990s, a significant breakthrough emerged with the identification of regulatory T-cells (Tregs), by Sakaguchi and colleagues following the work on tolerance induction by Hall (5–7). Sakaguchi et al. demonstrated that Tregs could be distinguished by the expression of the IL-2 receptor α-chain, CD25, which was exclusive to a minority of CD4$^+$ cells, facilitating the isolation of relatively pure Tregs populations from mice. The Shevach group provided the first direct evidence of Tregs inhibiting CD4$^+$ effector T-cell proliferation in culture (8). In 2001, several research groups independently succeeded in identifying Tregs in human peripheral blood utilizing CD25 (9–11). Given human conventional T-cells upregulate CD25 upon activation, it soon became evident that this early cell-surface marker had certain limitations, despite David Hafler's group demonstrating CD25 expression directly correlates with suppressive capacity (12).

The discovery of Foxp3, a forkhead box transcription factor, as the master control gene in mouse Treg development and function, provided further progress in the field and crucial insights into Treg regulatory mechanisms (13–15). It also explained the fatal lymphoproliferative disease observed in scurfy mice, which carry a frameshift mutation resulting in scurfin lacking the forkhead domain, and consequently autoimmunity (16). Intranuclear FOXP3 expression was demonstrated in human Tregs in 2005 (17), and mutations in FOXP3 explained the immune dysregulation, polyendocrinopathy, enteropathy and X-linked clinical syndrome (IPEX) (18, 19). Due to its intranuclear localization necessitating cell fixation, this new marker could not be used for the isolation of live Tregs. The discovery that FOXP3 expression could be induced in conventional human T-cells upon activation further complicated the identification of pure human Tregs (20).

In 2006, the expression of the IL-7 receptor α-chain CD127 was found to inversely correlate with FOXP3 expression and suppressive function (21, 22). In combination with CD25, low CD127 expression levels facilitated the isolation of live human Tregs with high purity and FOXP3 expression in post-sort analyses. Shortly thereafter, a more specific method to determine Treg purity among FOXP3 expressing cells was identified. Methylation analysis of the FOXP3 conserved non-coding sequence 2 (CNS2) (also termed the Tregs-Specific Demethylated Region (TSDR)) is at least partially methylated in conventional T-cells, but is completely demethylated in *bona fides* FOXP3$^+$ Tregs and remains the most specific method of identifying Tregs (23, 24).

Currently, CD3$^+$CD4$^+$CD25^{hi}CD127^{lo} remains the most widely used gating strategy for the isolation of viable Tregs when cell sorting, with intranuclear staining of FOXP3 used to confirm purity and function. Of note, however, FOXP3, whilst exclusive to Tregs in mice, does not provide unambiguous identification of human Tregs. Finding a specific marker for human Tregs remains an unmet need. Nevertheless, over the past two decades, a substantial number of novel Treg markers associated with their origin, maturity, stability, and function have been identified (Figure 1). This review gives a comprehensive analysis of human Treg markers, facilitating the precise identification of CD4$^+$ Tregs and their subsets, enabling the characterization of Tregs in a variety of immunological disorders and physiological processes.

2 Naïve and memory T-cell components as markers of Treg heterogeneity and functional diversity

Until 2005, Tregs in the peripheral blood were believed to predominantly exhibit a memory-like phenotype, characterized by the expression of CD45RO, until Valmori et al. described the existence of circulating Tregs expressing the naïve T-cell marker CD45RA (25). The prevalence of this subset inversely correlates with age, and concomitantly falls with the naïve CD4$^+$ T-cell fraction, whilst the total Treg frequency remains constant throughout life. Naïve Tregs also have longer telomeres than their memory counterparts, similar to naïve conventional CD4$^+$ T-cells. These naïve Tregs were postulated to be derived from CD4$^+$CD25$^+$ cells, selected in the thymus as precursors to antigen-experienced memory Treg subsets, awaiting TCR stimulation-mediated maturation, leading to an interest in umbilical cord blood Tregs.

A comparative study of CD45RA$^+$ naïve Tregs from umbilical cord blood and adult blood found slightly lower levels of CD25 and FOXP3 expression in both when compared to memory Tregs, but similar *in vitro* suppressive capacities to memory Tregs (26). In 2006, Hofmann and Edinger showed the CD45RA$^+$ naïve fraction of adult Tregs, which phenotypically resemble cord blood Tregs, gives rise to a more homogeneous Treg pool upon *in vitro* expansion as compared to bulk Tregs, thus proposing naïve Tregs as the optimal source of Tregs for adoptive cell therapies (27). They

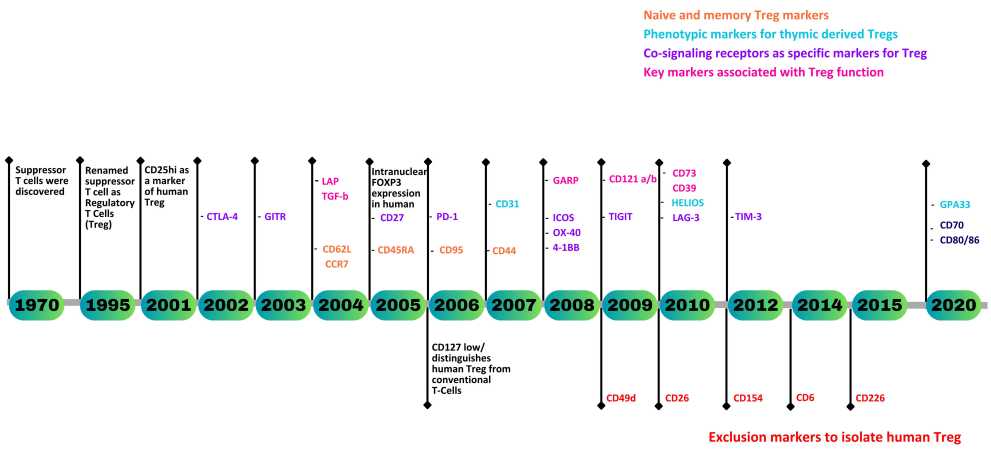

FIGURE 1
A timeline representing the discovery of human Treg markers.

also demonstrated the superior stability of $CD45RA^+FOXP3^+$ cells. However, after three weeks of *in vitro* expansion with repetitive stimulation, $CD45RA^-$ Tregs preferentially down-regulated FOXP3 expression and were liable to produce pro-inflammatory cytokines (28). Miyara et al. confirmed the enhanced proliferative capacity of naïve Tregs and found that once activated, naïve Tregs upregulate FOXP3 and convert to an activated memory phenotype, all whilst exerting suppression during and after their proliferation and conversion (24).

Furthermore, both adult naïve and cord blood Tregs were shown to express significant levels of the secondary lymph node homing receptors CD62L (L-selectin) and CCR7 (25, 26). These markers, along with CD45RA/RO are commonly used to distinguish between naïve ($CD45RA^+/RO^-$, $CD62L^{hi}$, $CCR7^{hi}$), effector memory (Tem) ($CD45RA^-/RO^+$, $CD62L^{lo}$, $CCR7^{lo}$), and central memory (Tcm) ($CD45RA^-/RO^+$, $CD62L^{hi}$, $CCR7^{hi}$) conventional $CD4^+$ T-cells. While studies have identified CCR7 as a differential marker defining Tem and Tcm subsets within the human $CD4^+CD25^+$ Treg population (29, 30), there is limited evidence for clear Tem and Tcm characteristics in Tregs based solely on these markers. Nevertheless, the expression of CD62L and CCR7 is crucial to the migration of Tregs to secondary lymphoid organs in murine models, proving essential to Treg-mediated suppression of autoimmune (30–33), allograft and other immune responses (34–38). Interestingly, even after undergoing extensive *in vitro* polyclonal expansion, human $CD4^+CD25^{high}$ Tregs retain the expression of CD62L and CCR7 (39). It would be intriguing to monitor CD62L and CCR7 expression in Tregs used for human cell therapy trials, examining if, and how, these markers change with Treg migration to secondary lymphoid organs and inflamed tissues.

In contrast to conventional $CD4^+$ T-cells, the majority of Tregs in adult peripheral blood exhibit elevated levels of CD45RO and CD95, making them susceptible to CD95L-mediated apoptosis (40). Interestingly, $CD4^+CD25^+FOXP3^+$ T-cells present in cord blood are predominantly naïve, display low expression of CD95 like adult naïve Tregs, and remain resistant to FAS-mediated apoptosis. However, upon brief stimulation with anti-CD3/CD28 monoclonal antibodies, cord blood Tregs significantly upregulate CD95, sensitizing them to CD95L (40).

The expression of CD44, a cell surface molecule with important roles in activation, migration, and apoptosis, is linked to FOXP3 expression and Treg function in mice (41). Similar studies in human Tregs indicated that CD44 enhances the suppressive action of $CD4^+CD25^+$ Tregs (42). Further investigation in 2009 demonstrated that CD44 co-stimulation plays a crucial role in enhancing the persistence and function of $FOXP3^+$ Tregs through the production of IL-2, IL-10, and TGF-β1 in both humans and mice (43).

3 Phenotypic markers of Tregs derived from the thymus

It was formerly believed that natural Tregs are thymically derived $CD4^+$ cells that constitutively display CD25. Work in mice demonstrating the capacity for naïve $CD4^+$ T-cells to transform into $FOXP3^+$ Tregs in the periphery led to a paradigm shift. Subsequent categorization of Treg subsets now includes thymically derived Tregs (tTregs) and peripherally induced Tregs (pTregs) (44). However, the presence of pTregs in humans remains a controversial topic. Multiple reports have indicated that pTregs are plastic and can produce inflammatory cytokines (45–47). Significant attention has been devoted to identifying phenotypic markers capable of distinguishing between pTregs and tTregs.

Helios (Ikzf2) is a member of the Ikaros transcription factor family and is restricted to T lymphocytes. Microarray studies characterizing unique gene signatures in FOXP3+ mouse Tregs provided the first indication of Helios as a Treg-specific gene (48, 49). In 2010, the Shevach group published work indicating Helios to be a potential marker specific to $FOXP3^+$ tTregs in mice and humans (50). Approximately 70% of Tregs in human peripheral blood and mouse peripheral lymphoid tissues, as well as over 95% of Tregs in mouse thymi, were Helios positive. Additionally, Helios expression was not seen neither in murine nor human *in vitro* or *in*

vivo induced Tregs. Interestingly, the majority of FOXP3$^+$ Tregs producing IL2, IL17, and IFN-γ belong to the Helios negative subset. Additionally, more than 90% of Tregs in human cord blood and thymic samples express Helios (51). Crucially, the Helios-negative Treg subset, comprising less than 10% of the total, does not originate exclusively from the thymus. Studies have suggested, in the case of cord blood, Helios-negative Tregs are generated in the periphery during fetal gestation, while those found in the thymus are recirculated Tregs from the periphery. Subsequent studies have demonstrated that human FOXP3$^+$Helios$^+$ Tregs demonstrate less than 10% CpG methylation in the TSDR, while FOXP3$^+$Helios$^-$ Tregs are more than 40% methylated (52, 53). Thus, the early consensus was Helios could serve as a marker for tTregs, with Tregs lacking Helios representing pTregs.

More recent studies have disputed this consensus (54, 55), demonstrating inducible Helios expression in pTregs (56), findig Helios expression with Treg activation and proliferation (57). The coexistence of both Helios positive and negative human FOXP3$^+$ tTregs has also been demonstrated (58), with further studies supporting the notion that Helios expression cannot differentiate between tTregs or pTregs (59). Thus, the question of whether Helios is a reliable specific marker for tTregs remains open (60).

A 2007 study by Hass et al. on Treg dysfunction in multiple sclerosis demonstrated a significant correlation between Treg-mediated immunosuppression and the presence of recent thymic emigrants (RTE) Tregs (61). Naïve Tregs expressing CD31 (PECAM1) play a role in the functional characteristics of the entire Treg population. CD45RA$^+$CD45RO$^-$CD31$^+$ naive Tregs have been observed to decline with age in healthy individuals, contrasting with their CD31$^-$ memory Tregs counterparts. Subsequent studies indicate that, unlike memory Tregs, a substantial proportion of CD45RA$^+$ naive Tregs express CD31 (62, 63).

A novel marker, Neuropilin-1 (Nrp-1), was identified in 2012 by Bluestone and Lafaille. It was found to be expressed on tTregs, but not on *in vivo* or *in vitro* induced pTregs in mice (64, 65). While these murine studies are useful to our understanding of Treg development and putative tTreg biomarkers, the translation of these findings into the human context remains disputed (59, 66, 67).

Glycoprotein A33 (GPA33) is a cell surface antigen and a member of the immunoglobulin superfamily. While this marker is known to be expressed in approximately >95% of primary and metastatic colon cancers, its presence on normal tissues and cells was previously unreported (68). In a 2020 study, Opstelten et al. identified GPA33 on tTregs, reporting high levels of mRNA and surface protein expression in CD4$^+$CD25$^+$CD45RA$^+$ naive Tregs (69). All CD4$^+$CD25$^+$CD127$^-$GPA33$^+$ Tregs were shown to express FOXP3 and Helios, whereas only 86% of CD4$^+$CD25$^+$CD127$^-$CD45RA$^+$ expressed FOXP3 and Helios, suggesting CD127$^-$GPA33$^+$ selects naïve Tregs with enhanced purity, lineage stability, and suppressive function. Subsequent single-cell transcriptomic studies have demonstrated that GPA33 is acquired by pre-Tregs in a late developmental stage, prior to the acquisition of CD45RA, validating GPA33 as a tTreg marker (70). Combined, this suggests GPA33$^+$ is found on a highly pure and stable population of CD45RA$^+$ Tregs originating from the thymus. It will be interesting to assess the applicability of this intriguing new marker for the isolation and expansion of functional Tregs, especially as the function of GPA33 is presently unclear.

4 Co-signaling receptors as markers of Treg proliferation and function

Co-stimulatory and co-inhibitory receptors, collectively described as co-signaling receptors, are crucial to regulating the expansion and function of conventional CD4$^+$ T-cells, modulating immune responses. Their discovery marked a turning point in our understanding of Treg function, eventually leading to various clinically employed therapeutic agents, called checkpoint inhibitors, which target Co-signaling receptors to restore desired immune responses (71, 72).

Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), also known as CD152, is an immunoglobulin superfamily (Ig SF) member expressed by activated inhibitory conventional T-cells (73). CTLA-4 is often referred to as a "moving target" due to its rapid internalization and recycling to the plasma membrane of T-cells (74). Notably, while CTLA-4 is expressed in conventional T-cells upon activation, it is constitutively expressed in resting Tregs, representing one of the hallmarks of this cell subset (75, 76). Post TCR-mediated activation, human Tregs strongly upregulate CTLA-4.

In 2000, work by the Powrie and Sakaguchi groups provided compelling evidence of CTLA-4's crucial role in contact-dependent suppressive function in mice (76, 77). Subsequent human Treg *in vitro* studies demonstrated conflicting results. Some supported the notion that blocking CTLA-4 using anti-CTLA-4 monoclonal antibodies (mAbs) or Fab fragments reversed the suppressive effect of CD4$^+$CD25$^+$ Tregs on Teff proliferation (78–80). Other studies came to the opposite conclusion (9, 10, 81, 82), highlighting the complex nature of CTLA-4's role in Treg-mediated suppression. However, variations in assay conditions, including the type of antigen-presenting cells (APCs) used and the strength of the TCR stimulus may account for the differing results observed. CTLA-4 interacts with CD80/CD86 on APCs with higher affinity and avidity than its counterpart CD28, which competes to interact with CD80/86. As a target gene of FOXP3, CTLA-4 plays a pivotal role in regulating Treg homeostasis by acting as an intrinsic regulator of Treg proliferation. Deletion or blockade of CTLA-4 enhances Treg proliferation and affects the number and function of memory Tregs (83–86). Although the role of CTLA-4 in Treg biology has remained controversial for several years, its established use as a diagnostic marker and therapeutic target for various human pathologies confirms its critical role in Treg-mediated immunosuppression (72, 87–89).

In parallel to CTLA-4, programmed cell death 1 (PD-1) is another co-inhibitory molecule from the Ig SF found in Tregs. In 2003, it was found that blocking the interaction between PD-1 and its ligand, PD-L1, influences Treg suppressive function (82). A more comprehensive 2006 study demonstrated that freshly isolated murine and human Tregs retain PD-1 in the intracellular compartment (90). PD-1 appears to transmigrate to the cell

surface upon TCR stimulation, indicating that purifying CD25^{+}PD-1^{-} T-cells may give a pure population of resting Tregs, prompting evaluation of PD-1 as a marker to distinguish Tregs from activated CD4+CD25+ T-cells. Francisco et al. showed that the expression of PD-1 minimally converts naive CD4^{+} T-cells into induced Tregs (iTregs) by promoting FOXP3 expression, enhancing iTreg suppressive activity (91). Our current understanding of PD-1/PD-L1 signaling in Tregs is complex and context-dependent, with effects including Treg expansion (92), survival and functionality (93) in addition to suppression of effector T-cells and autoreactive B-cells (94). These effects have implications for tumor immunity and autoimmunity, as reviewed by Cai et al. (95) and the clinical use of PD-1 inhibitors highlights the therapeutic value of targeting this receptor. Nevertheless, further research deciphering the intricate mechanisms of PD-1 signaling in Treg development and function is required.

Inducible T-cell costimulator (ICOS) is another CD28-related member of the immunoglobulin superfamily of molecules (*IgSF*) that stands apart from CTLA-4 and PD-1 by functioning as a costimulatory receptor. ICOS's discovery on the surface of T-cells following TCR-mediated stimulation in 1999 represented a breakthrough in T-cell research (96). ICOS^{+} T-cells, although a minority among peripheral blood CD4^{+} T-cells, comprise approximately 20% of FOXP3^{+} Tregs (97). Ito et al. observed that both ICOS^{+} and ICOS^{-} Tregs exist in cord blood and neonatal thymi (98) and that ICOS^{+} Tregs are able to produce IL-10 and suppress CD86 upregulation on DCs, while ICOS^{-} Tregs produce TGFβ. Early investigations unveiled a link between elevated ICOS expression on CD4^{+} T-cells and IL-10 production in mice (99). This was later confirmed in humans (98) and demonstrated the central role ICOS has in the differentiation and function of FOXP3^{+} Tregs. Over the past decade, ICOS's multifaceted roles, including involvement in the production, proliferation and survival of Tregs and enhancing Treg suppressive function have been unraveled (100–103).

CTLA4, PD1 and ICOS are now recognized targets in cancer immunotherapy, leading to an interest in novel Ig SF co-inhibitory receptors such as Lymphocyte activation gene-3 (Lag-3), T-cell (or transmembrane) immunoglobulin and mucin domain 3 (Tim-3), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT), to address CTLA-4 and PD-1 checkpoint inhibitor non-responders.

Lag-3, also called CD223, belongs to the CD2/Signaling Lymphocytic Activation Molecule (SLAM) family of the Ig SF. It was initially discovered as a molecule upregulated on activated CD4^{+} and CD8^{+} T-cells (104) and has subsequently been reported to be highly expressed in both tTregs and pTregs upon activation (105). Lag-3 blockade on Tregs abolishes Treg suppressor function, while ectopic expression of Lag-3 in non-Treg CD4^{+} T-cells confers suppressive activity. Additionally, Lag-3 is necessary for Treg-mediated control of T-cell homeostasis (106). In subsequent years, Lag-3 has emerged as a useful Treg surface marker that can be cleaved into a soluble form (107, 108). Functional studies of Lag-3 in human Tregs report only small proportions of Tregs are LAG-3 positive, although this fraction increases in inflamed tissues such as in the lamina propria of ulcerative colitis (UC) patients, and various tumors (109–111). Thus, Lag-3 may serve as a diagnostic and treatment response biomarker in the future, however further research into the mechanism of action of Lag-3 in Tregs and its impact on T-cells is warranted.

TIGIT, a member of the poliovirus receptor (PVR)-like family of Ig SFs, is a co-inhibitory receptor expressed on many lymphocyte subsets. It is highly expressed on Tregs, where it promotes suppressive function. The first link between TIGIT and human Treg biology was reported in 2009 (112), however, its functional role in Tregs was not shown until 2014 (113). TIGIT is present on the surface of both human and murine Tregs, including tTregs and pTregs. TIGIT-positive Tregs exhibit increased expression of key Treg-associated genes, including FOXP3, CD25, and CTLA-4, and demonstrate enhanced demethylation in the TSDR, indicating lineage stability. Activation of TIGIT on Tregs triggers the production of suppressive molecules like IL-10 and Fgl2. Moreover, TIGIT expression defines a Treg subset that demonstrates selective suppression of Th1 and Th17 but not Th2 responses. Additional studies have reported TIGIT to identify highly suppressive Tregs, indicating the therapeutic potential of TIGIT Tregs in treating disease (114–116).

Tim-3 (HAVCR2) has attracted significant attention as a potential negative regulator of T-cell responses (117, 118). Tim-3 expression is limited to a small subset (2-5%) of Tregs in the periphery during steady-state conditions. However, during immune responses, Tim-3 is upregulated on Tregs, with elevated expression in Tregs infiltrating allografts, tissues, and tumors in mice (119). Subsequent studies have found that Tim-3 is predominantly expressed on tumor-infiltrating Tregs in both human and experimental tumor models (120–126), and Tim-3^{+} Tregs exhibit enhanced suppressive capacity in *in vitro* suppression assays compared to Tim-3^{-} Tregs (122, 124). Moreover, Tim-3^{+} Tregs display increased expression of suppressive molecules (CTLA-4, Lag-3, and PD-1), and enhanced secretion of immunosuppressive cytokines (IL-10 and TGF-β). These, and more recent findings, strongly argue for Tim-3^{+} Tregs cells as promising therapeutic target in cancer immunotherapy (127). Furthermore, a substantial increase in Tim-3 levels are seen following the *ex vivo* expansion of clinical-grade human Tregs, increasing to 29% (128). Sorted Tim-3^{+} Treg populations show significant enhancements in IL-10 and Granzyme B production, indicating the suppressive capacity of this subset and its potential as a cell-based therapy to induce allograft tolerance.

The TNF receptor superfamily also includes several co-stimulatory molecules that have been identified on Tregs, including GITR, 4-1BB, OX40 and CD27.

Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), also known as TNFRSF18 and CD357, acts as a costimulatory molecule. While expressed at low levels on naïve T-cells, Tregs and activated T-cells show high surface expression of GITR (129, 130). Soon after the discovery of GITR on murine Tregs (131, 132), studies reported GITR to be co-expressed with CD25 and FOXP3 in human tTregs (133–135). GITR is now an established Treg marker with tTreg specificity, whose gene loci is demethylated in Tregs (136). Tregs upregulate GITR expression upon activation, with levels correlating with Treg immunosuppressive function, leading to the proposal of using

GITR as a marker for selecting highly functional Tregs. Additionally, Tregs residence in the tumor microenvironment demonstrates GITR upregulation and GITR's expression in Tregs has been extensively studied in autoimmune diseases, demonstrating its potential as an immunotherapy target having already shown promise in murine models (137, 138).

4-1BB (also known as CD137), was initially considered a proliferation and activation marker of $CD8^+$ conventional T-cells, but was later found to be constitutively expressed in murine $CD4^+CD25^+$ Tregs in two separate DNA microarray studies (132, 139). 4-1BB expression on resting Tregs was subsequently identified, with upregulation demonstrated following 2 days of *in vitro* combined CD3 and IL-2 stimulation (140). Subsequently, in both *in vitro* and *in vivo* conditions, 4-1BB mediated enhanced Treg survival and proliferation was observed, without alteration to immunosuppressive function (140, 141). Additionally, animal studies have shown infusions of agonistic anti–4-1BB mAbs lead to increases in splenic Tregs, with suppression of chemically induced colitis (142). Furthermore, artificial APCs (aAPCs) expressing 4-1BBL have been shown to expand human umbilical cord blood Tregs and enhance their suppressive function (143). Most significantly, it has been found that gating on $4\text{-}1BB^+CD40L^-$ ($CD137^+CD154^-$) identifies both *ex vivo*, and *in vitro* activated Tregs, facilitating the isolation of epigenetically stable antigen-activated Tregs, enabling their rapid functional testing (144, 145).

Along with the discovery of 4-1BB on Tregs, the aforementioned DNA microarray studies also identified OX40 (CD134) to be highly expressed on Tregs, which had previously been recognized as a costimulatory molecule on activated $CD4^+$ effector T-cells (132, 139). OX40 signaling is crucial to the development, homeostasis, and suppressive activity of mouse Tregs (146). Early studies of human cord blood Tregs demonstrated significantly higher Treg expansion using aAPCs modified to express OX40L compared to bead- or non-modified aAPCs (143). However, OX40 signaling has also shown the potential to oppose Treg-mediated suppression in antigen-engaged naive T-cells in *in vivo* mouse models (146). The contrasting functions of OX40 in Tregs, which has also been reported in a variety of human and mouse studies, make its exact role in Tregs unclear (147–151). While uncertainty continues regarding OX40 in Tregs, it remains recognized as a Treg activation marker and is believed to contribute to Treg proliferation.

CD27 is present on the majority of $CD4^+$ T-cells, interacting with CD70 on APCs, and *in vitro* T-cell expansion can be enhanced using anti-CD27 antibodies (152). Initially discovered through DNA microarray studies (132), CD27 is now recognized for its crucial role in Treg function. Along with CD25, CD27 can identify highly suppressive $FOXP3^+$ Tregs (153) and subsequent investigations have illustrated a novel use of CD27 to induce, expand, and select highly suppressive, Ag-specific human Treg subsets (154). Moreover, following the *ex vivo* expansion of human pTregs, CD27 can discriminate between regulatory and non-regulatory cells (155). With multiple studies highlighting CD27 as a reliable marker for the identification of highly functional Tregs (156, 157), CD27 demonstrates significant potential as a marker for the isolation of suppressive Tregs for clinical application.

Potential markers of Tregs exhaustion: Whilst the effects of chronic T-cell stimulation have been extensively studied, our understanding of Treg exhaustion is limited. In conventional T-cells, exhaustion from chronic antigen exposure is characterized by the expression of inhibitory receptors (PD-1, TIM-3, and LAG-3), reduced proliferation, cytokine production, and increased apoptosis (158–161). Studying Treg exhaustion *in vitro* is hampered by the contradictory roles of inhibitory receptors in Tregs, as discussed above. Tonic-signaling chimeric antigen receptors (TS-CARs) have facilitated the first comprehensive investigation of exhaustion in Tregs, revealing that Tregs rapidly acquire an exhaustion-like phenotype, with increased expression of inhibitory receptors and transcription factors including PD-1, TIM3, TOX and BLIMP1, akin to conventional T-cells (162). Epigenetic changes can also be observed with repetitive TCR-mediated stimulation, including those that may affect Treg functionality and lead to exhaustion, with TIM-3 and TIGIT implicated, among others (163). However, further clarification is needed on the implications of these recent studies on these markers for identifying exhausted Tregs.

5 Exclusion markers for human Treg isolation

One of the major obstacles hampering the clinical application of Tregs is the lack of suitable extracellular markers, complicating their identification and isolation. Due to a lack of clinical-grade CD4 negative isolation reagents, clinical grade GMP magnetic enrichment typically involves CD8 depletion, an optional CD19 depletion step, followed by CD25 enrichment. Where cell sorting is possible, Tregs are typically isolated as $CD4^+CD25^{+/hi}CD127^{-/low}$. Over the past decade, numerous studies have attempted to identify additional negative selection markers to facilitate the efficient sorting of pure and highly functional Tregs, while eliminating contaminating effector T-cells. In this section, we explore cell surface markers that are specifically absent on Tregs and consider negative selection strategies for Treg isolation for both functional research and clinical application.

CD49d, the α chain of the integrin VLA-4 (α4β1), functions as a costimulatory and adhesion molecule for lymphocyte transmigration into inflamed tissues (164, 165). In 2009, Kleinewietfeld et al. demonstrated CD49d expression on more than 80% of human PBMCs, with expression predominantly on pro-inflammatory effector cells, including non-regulatory $CD4^+CD25^{low}$ T-cells, cytokine-secreting $CD4^+$ effector cells, and FOXP3 expressing Th1- and Th17-like cells. Significantly, approximately 70% of immunosuppressive $FOXP3^+$ Tregs are $CD49d^{-/low}$. Considering the differential distribution of CD49d, this marker has been proposed to effectively deplete cells that otherwise contaminate $CD25^+$-based Treg preparations (166). This has led to novel $CD49d^-$ gating strategies, designed to obtain "untouched" $FOXP3^+$ Tregs (i.e. cells that have not been targeted by an antibody during purification) in combination with $CD127^-$. Tregs isolated in this manner are highly pure, can be expanded, and show effective *in vitro* and *in vivo* suppressive function (166). However, other data has shown $CD49d^-CD127^-$ isolated Tregs after

in vitro expansion are less suppressive, exhibit lower levels of FOXP3 and TSDR demethylation than $CD4^+CD25^{hi}CD127^-$ or $CD4^+CD25^{hi}ICOS^+$ isolated cells (167). Despite this conflicting evidence, untouched Treg isolation protocols have been scaled up for the generation of GMP cell therapy products from large-scale leukapheresis samples, with the final product demonstrating <10% contamination with CD4 effector T-cells and <2% of all other cell types (168). Singapore General Hospital is currently preparing to conduct T-cell therapy trials using untouched Tregs in graft-versus-host disease following stem cell transplantation.

Another putative negative selection marker for Tregs is CD26, a widely distributed, 110-kDa, membrane-bound glycoprotein with intrinsic dipeptidyl peptidase IV activity. The ectonucleotidases, CD39 and CD73, in combination with adenosine deaminase (ADA), which degrades adenosine into inosine, together regulate pericellular adenosine concentrations. In humans, ADA is associated with the extracellular domain of CD26, a complex not seen in mice (169). The lack of anti-ADA antibodies suitable for flow cytometry necessitates CD26 analysis as a surrogate for ADA expression in T-cells. Despite a large amount of data supporting the costimulatory role of CD26 in T-cells (170–172), its exact function in Tregs has not been confirmed. The absence of CD26-ADA expression in Tregs was first described in 2010 (173). More comprehensive work in 2012 reported the presence of CD26-ADA on FOXP3-expressing activated $CD4^+$ Teff cells, but not suppressive Tregs (174). In combination with markers like CD25, FOXP3 and CD127, CD26 may facilitate quantitative evaluation of Tregs or Treg isolation from samples containing activated Teff cells. Other studies have also described the use of CD26 as a negative selection marker for Tregs (175–178). However, further investigation is required to determine whether the $CD26^{-/low}$ phenotype is a reliable method of isolating human Tregs and one that improves on current protocols.

CD6 and CD226 have not only facilitated the depletion of contaminants but also shed light on further subclassifications of tTregs. CD6 is a cell-surface glycoprotein predominantly expressed on T-cells (179, 180). It functions as a costimulatory molecule during TCR activation and plays a role in the responses of mature T-cells to both nominal antigens and autoantigens (181, 182). In 2014, Garcia Santana et al. identified $CD4^+CD25^{hi}CD6^{lo/-}$ Tregs in man as $FOXP3^+$ natural Tregs (nTregs), exhibiting *in vitro* suppressive activity on $CD8^+$ T-cell proliferation. CD6 in combination with CD127 was postulated to serve as a tool to identify and isolate nTregs (183). Although CD6 has been reported as a negative marker for human Tregs in different studies, further investigation is needed to validate the utility of CD6 as a Treg exclusion marker (175, 184).

Although CD226, also known as DNAM-1, plays a critical role in immunoregulation, little was known about its cellular distribution in human Tregs until 2015, when Fuhrman et al. demonstrated that isolating $CD226^-$ Tregs gave a highly pure and suppressive population. The $CD226^+$ population is less pure and suppressive after expansion and demonstrated less demethylation in the TSDR locus, as well as significantly higher production of effector cytokines (185). Further work showed $CD4^+CD25^+CD226^-$ cells, after 14 days of expansion, are more suppressive, produce more TGF-β1 and fewer effector cytokines like IFN-γ, TNF, and IL-17A than $CD4^+CD25^+CD127^{lo/-}$ cells (186). Furthermore, $CD4^+$ $CD25^+$ $CD226^-$ Tregs may not only be long-lived but also potentially localize more readily to secondary lymphoid organs. These data argue for CD226 as an important negative phenotypic marker of Tregs and the excluding CD226-expressing cells during Tregs sorting yields a population with increased purity, lineage stability, and suppressive capabilities, which may benefit Treg adoptive cell therapy. However, this approach should be taken with caution, as CD226 is also highly expressed by IL-10-secreting Tr1-like T-cells (187).

In addition to the aforementioned, CD154, also known as CD40L, can also be used to negatively identify activated Tregs. This member of the TNF superfamily is a recognized activation marker of $CD4^+$ Teff cells (188, 189). The absence of CD154 expression in the presence of 4-1BB ($4\text{-}1BB^+CD154^-$) has emerged as an *ex vivo* and *in vitro* Treg activation signature, allowing for the identification and isolation of epigenetically stable, antigen-activated Tregs for rapid functional assessment (144, 145).

6 Key markers associated with the Treg function

Tregs exert their suppressive effects via a plethora of mechanisms, acting on various targets. These include modulating the cytokine microenvironment (85, 190, 191), metabolic disruption of target T-cells (8, 192), regulating the activation capacity of dendritic cells (193, 194), and direct cytolysis (195, 196). Building upon this, we next review previously discovered and newly identified markers of Tregs function.

Transforming growth factor-β (TGF-β), the most common isoform of which is TGF-β1, is a pleiotropic cytokine. First discovered in 1986, it was noted to induce anchorage-independent growth (197). TGF-β later became the primary focus of the immunosuppression and tolerance research field because of its potent inhibition of immune responses, impacting particularly upon T-cell proliferation and differentiation (198, 199). This was solidified when the connection between TGF-β and the suppressive function of murine Tregs was identified (77, 200), and the concept of TGF-β1 tethered to the cell surface of Tregs was introduced. Upon stimulation, $CD4^+CD25^+$ Tregs but not $CD4^+CD25^-$ conventional T-cells express high and persistent levels of TGF-β1 on the cell surface. However, the role of TGF-β in the immunoregulatory function of Tregs has sparked controversy, as previous studies had shown that neutralizing TGF-β using anti-TGF-β antibodies failed to reverse the suppressive function of Tregs *in vitro*. This was addressed in 2004 when it was reported that Latency associated protein (LAP) binds latent TGF-β1 in Tregs (201). Thus, TGF-β1-mediated immunosuppression occurs in a two-step process: first, TGF-β1 dissociates from LAP, then free TGF-β1 can interact with its receptor. This multistep process explains why antibody mediated TGF-β inhibition requires the high antibody concentrations necessary for TGF-β1 quenching. Crucially, expression of LAP on activated human $FOXP3^+$ Tregs

has since been shown to identify highly pure and functional Tregs from *ex vivo* expansion cultures (202, 203). For reviews on TGF-β in Treg biology, and the associated debates, see reviews by Tran and Moreau et al. (204, 205).

The cell surface molecule Glycoprotein A repetitions predominant (GARP), also known as LRRC32, was first reported in human Tregs by Wang et al. and has since been identified to be involved in TGF-β1 mediated Treg immunosuppression. TCR-activated Tregs exhibit significant upregulation of GARP on their cell surface, which has been linked to enhanced Treg suppressive capacity (206). Several groups have independently reported that GARP on Tregs binds latent TGF-β1, promoting the release of the activated TGF-β, leading to TGF-β mediated immunosuppression (207–209). Studies of Tregs in human disease have confirmed that TGF-β, GARP and LAP are vital to Treg function in inflammatory diseases (210) and cancers (211), highlighting the potential value of these receptors as immunotherapy targets.

Tregs can also suppress effector T-cells through adenosine binding to A2A receptors (212, 213). ATP is cleaved in tandem by two Treg-associated ectonucleotidases, CD39 and CD73, leading to adenosine production. Of the two ectonucleotidases, CD39, which hydrolyses ATP and ADP into AMP, is the rate-limiting enzyme (212). CD73, an ecto-5′-nucleotidase, exists in both soluble and membrane-bound forms and catalyzes the dephosphorylation of AMP into adenosine (214, 215). Adenosine then binds the A2A receptor on T-cells, leading to cAMP-mediated suppression of TCR signaling via PKA.

The role of adenosine in the function of human Tregs was discovered in 2010, introducing CD39 as a novel phenotypic and functional marker specific to human Tregs (173). In contrast to mice, which express CD39 constitutively on virtually all $CD4^+CD25^+$ T-cells, expression on human T-cells is restricted to a subset of FOXP3+ effector/memory-like Tregs (216, 217). Nearly all (>90%) $CD4^+CD25^{hi}FOXP3^+$ adult human Tregs are CD39 positive, distinguishing human Tregs from other T-cell subsets. Due to its expression on the cell surface, CD39 has facilitated the successful and reliable isolation of functionally active human natural Tregs from peripheral blood (218). However, this approach should be taken with caution, as CD39 is not exclusive to Tregs and the $CD4^+CD39^+$ fraction of PBMCs also includes $CD25^-FOXP3^-$ T-cells (219, 220). Nevertheless, several studies have revealed that $CD39^+$ Tregs demonstrate stronger stability and function under inflammatory conditions and superior suppressive capacity *in vitro* and *in vivo* (221–224), as well as an ability to suppress pathogenic Th17 responses (225).

CD73 was initially described as a characteristic surface marker of murine Tregs, however, it was only observed in a small proportion of human Tregs. Over 70% of $CD4^+CD25^{hi}$ Tregs express CD73 intracellularly, while the expression is limited to 20% of CD4+CD25- T-cells (173). These data indicate that CD73 is readily internalized from the surface of human lymphocytes (226, 227) and is predominantly cytosolic in human Tregs. However, expanded human $CD4^+CD25^{hi}CD127^{lo}$ cells show a higher surface expression of CD73 (~35%) as compared to unstimulated memory Tregs ($CD4^+CD25^{hi}CD127^{lo}CD45RA^-$) (~5%) (228). These expanded Tregs are highly suppressive, attributable to the surface expression of CD73 along with CD39. Additionally, CD73 on murine Tregs has been shown to suppress proliferation and cytokine secretion by T helper 1 (Th1) and Th2 cells (229). Taken together, these results strongly indicate the physiological importance of CD39 and CD73 expression in Tregs (228, 230).

7 Emerging markers of Tregs function

As previously discussed, a multitude of Treg markers are associated with their origin, maturation, stability, and functional characteristics, as concisely delineated in Figure 2. In recent years, several emerging markers have garnered attention for their potential contributions to Treg-mediated immunosuppression. Although the precise roles and mechanisms of action remain a subject of ongoing investigation, preliminary research suggests their potential significance in modulating immune responses.

CD121a and CD121b are two unique cell-surface antigens on human Tregs that are transiently expressed upon TCR-mediated Treg activation, distinguishing functional Tregs from activated $FOXP3^-$ and $FOXP3^+$ non-Tregs, alongside LAP (202). These two highly specific surface markers were recommended for high yield, high purity Treg isolation, promising a rapid advancement in the therapeutic application and functional analysis of Tregs in human disease. However, the popularity of these two markers has waned due to a lack of subsequent studies that could verify these properties.

CD69 and HLA-DR are widely recognized as activation-induced cell surface markers in both conventional T-cells and Tregs, as well as markers of Treg differentiation and immunosuppression. CD69, upon binding with its ligand the S100A8/S100A9 complex, regulates Treg differentiation (231). Further reports indicate CD69 expression may be essential for tTreg development (232). Moreover, $FOXP3^+CD69^+$ Tregs express higher surface levels of suppression-associated markers and display enhanced suppressive activity as compared to $FOXP3^+CD69^-$ Tregs (233, 234). CD69 also enhances the immunosuppressive function of Tregs by prompting IL-10 production (235). However, due to the lack of knowledge regarding the functional roles of CD69 in human Tregs, no definitive conclusions can be drawn at this time.

Approximately one-third of adult human peripheral blood CD4 + effector Tregs express HLA-DR, identifying a distinct and highly functional subset of terminally differentiated Tregs (236). $HLA\text{-}DR^+$ Tregs exhibit higher levels of FOXP3 and employ contact-dependent immunosuppression, indicating superior suppressive capabilities (237, 238). However, despite HLA-DR+ Tregs being regarded as superior in functionality (239–241), there is insufficient data demonstrating HLA-DR as a marker of Treg function.

Two other cell surface receptors, CD101 and CD129, have been linked with Treg suppressive activity (242). Earlier mouse studies revealed that CD101 surface expression is strongly correlated with suppressive activity in $CD4^+CD25^+$ Tregs, both *in vitro* and *in vivo* (243). However, studies in human Tregs have not replicated this observation (244). Similarly, IL-9 generated by activated T-cells promotes the proliferation of Th clones and enhances the suppressive function of CD129 (the IL-9 receptor) expressing Tregs (245, 246). However, these two markers have lost popularity given the lack of evidence for them in man.

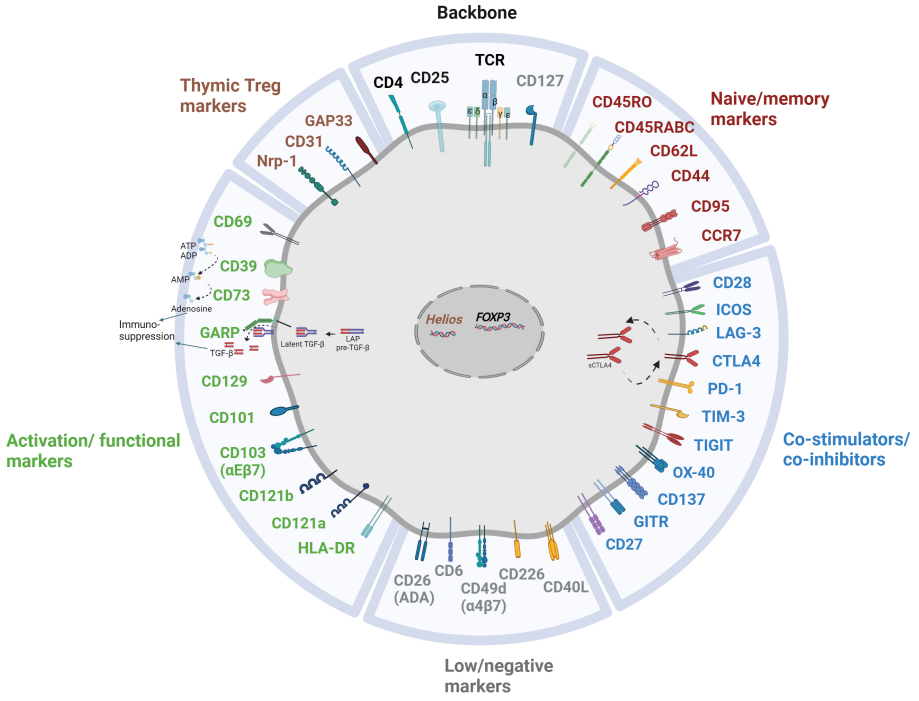

FIGURE 2
Multitude of markers illuminating the heterogeneity of Treg subpopulation.

CD103, also known as integrin αEβ7 (ITGAE), is expressed on approximately 10-30% of mouse pTregs. Whilst not indicative of activation status, its levels can be upregulated in inflamed tissues (247–249). $CD103^+$ Tregs have been found to exhibit slightly higher suppressive capacity and express higher levels of IL-10, contributing to their anti-inflammatory activity (132). Other findings in mice include the ability of $CD103^+$ Tregs to restrain $CD8^+$ T-cell activation (250). Human $CD25^+FOXP3^+$ Tregs, however, show limited expression of CD103 in various tissues, including blood (<5%) (251–254). Nevertheless, human CD4 T-cells can be induced to express CD103 through various stimuli *in vitro* (255, 256) and the percentage of $CD103^+$ cells among $CD4^+$ $CD25^+$ T-cells is significantly higher than $CD4^+CD25^-$ T-cells in patients with multiple sclerosis (251). Further investigation is needed to determine whether CD103 is upregulated on human Tregs in other inflammatory conditions.

Recent studies have identified the potential expression of CD70 (157), CD80, and CD86 (257) in Tregs, opening new possibilities for identifying novel Tregs subsets and markers of their potential therapeutic efficacy.

The summary of all the markers discussed above with their alternative designations, and their patterns of expression in human Tregs is presented in Table 1.

8 The functional role of chemokines and chemokine receptor interactions in Treg migration

Chemokines are a group of small heparin-binding proteins that direct the movement of circulating immune cells, influencing their migration within inflamed tissues (258, 259). When initially identified, chemokines were described to be associated with inflammatory diseases (260–262). Later, their involvement in the migration of immune cells was identified (263), followed by production by immune cells themselves (264, 265). A plethora of studies have shown how perturbations in the distribution of Tregs can lead to organ-specific inflammatory diseases (258, 266–275). Chemokines govern the trafficking and homing of Tregs, and understanding how Tregs reach their site of action sets the ground for targeting the pathogenic Treg distribution in the context of cancer and may facilitate tissue-specific targeting in the context of Treg therapy. Variations in chemokine expressions across different human organs is summarized in Figure 3.

Several integrins have been identified to be crucial to Treg function and localization. The αvβ8 integrin is key to activating latent TGF-β and Tregs lacking αvβ8 are unable to suppress T-cell-mediated inflammation *in vivo* (276, 277). The α4β7 integrin (also known as LPAM-1 and whose classical ligand is MAD-CAM1) is recognized to play a crucial role in the migration of Tregs to the intestine and associated lymphoid tissues. Loss of the β7 has been shown to lead to disrupted migration, with subsequent colitis observed (266, 278, 279). The expression of the α4β7 integrin and CCR9 enables Tregs to home to the gut's lamina propria, allowing for tolerance to food antigens, crucial to maintaining gut health (266, 267).

8.1 Tregs in lymphoid tissue

Tregs play a major role in constraining immune response in secondary lymphoid organs, with CCR7 being one of the first

10.3389/fimmu.2023.1321228

TABLE 1 Markers of human functional FOXP3$^+$ Tregs.

Markers	Alternative name(s)	Marker location	Expression on resting Treg	Expression level upon activation
CD25	IL-2Rα	Surface	CD25^{high}	Very high
FOXP3	N/A	Intranuclear	FOXP3^{high}	Very high
CD127	IL-7Rα	Surface	CD127$^{low/-}$	Low/-
CD45RA	N/A	Surface	Naïve Treg are predominantly CD45RA$^+$	Low/-
CD45RO	N/A	Surface	Memory Treg are predominantly CD45RO$^+$	High
CD62L	L-selectin	Surface	Treg with secondary lymph node homing capacity are predominantly CD62L$^+$	Retained
CCR7	CD197	Surface	Most of the Treg with secondary lymph node homing capacity are CCR7$^+$	Retained
CD95	Fas	Surface	Most of the naïve Treg are CD95$^{low/-}$	High
CD44	N/A	Surface	Most of the Treg are CD44$^{low/-}$	High
CD31	PECAM-1	Surface	Recent thymic emigrant Treg are CD31$^+$	Low
Helios	IKZF2	Intranuclear	Thymus derived stable Treg are Helios$^+$	Stable over time
GPA33	A33	Surface	Pure and stable naïve Treg population, that are also derived from thymus, are GPA33$^+$	low
CTLA-4	CD152	Surface/ Intracellular	Treg express CTLA-4 constitutively at low level	High
PD-1	CD279	Surface/ Intracellular	Most of the PD-1 expressing Treg retain its expression in the intracellular compartments	High (surface)
ICOS	CD278	Surface	About 20% of the peripheral Treg are ICOS$^+$	High
TIGIT	VSTM3	Surface	Few Treg with activated phenotype are TIGIT$^+$	High
LAG-3	CD223	Surface	Most of the resting Treg are LAG3$^{low/-}$	High
TIM-3	CD366 HAVCR2	Surface	Only 2-5% of Treg in periphery are TIM-3$^+$	High
GITR	TNFRSF18 CD357	Surface	Few Treg with activated phenotype are GITR$^+$	High
OX-40	CD134	Surface	Few Treg with activated phenotype are OX-40$^+$	High
4-1BB	CD137	Surface	Few Treg with activated phenotype are 4-1BB$^+$	High
CD27	N/A	Surface	Treg with high suppressive capacity are CD27$^+$	Mostly stable over time
CD49d	α4 integrin VLA-4 α	Surface	70% of the immunosuppressive FOXP3+ Treg are CD49d$^{low/-}$	High
CD26	ADA binding protein	Surface	Most of the Treg are CD26$^{low/-}$	High
CD6	–	Surface	Most of the Treg are CD6$^{low/-}$	High
CD226	DNAM-1	Surface	Most of the Treg are CD226$^{low/-}$	High
TGF-b	TGF-b1, LAP	Surface/ Intracellular	Most of the resting Treg are TGF-b$^{low/-}$	High
LAP	TGF-b1	Surface/ Intracellular	Most of the resting Treg are LAP$^{low/-}$	High
GARP	LRRC32	Surface	Most of the resting Treg are GARP$^{low/-}$	High
CD39	N/A	Surface	Only effector/memory-like Treg are CD39$^+$	High
CD73	N/A	Surface/ Intracellular	Most of the CD73 expressing Treg retain in the intracellular compartments	High (surface)
CD121a/b	IL-1R I/II	Surface	Most of the resting Treg are CD121a/b$^{low/-}$	High (transient)

(Continued)

TABLE 1 Continued

Markers	Alternative name(s)	Marker location	Expression on resting Treg	Expression level upon activation
CD69	N/A	Surface	Most of the resting Treg are $CD69^{low/-}$	High
HLA-DR	N/A	Surface	Few Treg with activated phenotype are $HLA\text{-}DR^{+}$	High
CD101	BB27	Surface	Less studied in humans	N/A
CD129	IL-9R	Surface	Less studied in humans	N/A
CD103	Integrin αEβ7	Surface	<5% Treg in various tissues, including blood are $CD103^{+}$	N/A
Neuropilin-1	CD304	Surface	Thymic derived mouse Treg are Neuropilin-1^{+} (Human studies are controversial)	N/A
CD70	CD27L	Surface	Only 5-6% Tregs are $CD70^{+}$	High
CD80/ CD86	B7 1/2	Surface	20% Treg are $CD80^{+}$ and <1% Treg are $CD86^{+}$	High

References are indicated in the text.
The table above presents an overview of human Treg markers that delineate different subsets of Treg cells, along with their respective properties, as detailed in the review.

chemokine receptors identified on Tregs (30, 280). $CD62L^{hi}CCR7^{+}$ naïve Tregs favor migration into secondary lymphoid organs where professional antigen presentation allows for antigen-dependent stimulation, modifying the pattern of receptor expression to enable tissue homing (30, 30, 33, 281, 282). Lymph nodes draining these organs act with local dendritic cells to induce Treg activation and action, while CCR7 is downregulated, promoting accumulation in inflamed tissues (38, 283–285). Notably, the loss of CCR7 results in impaired *in vivo* Treg suppressive activity, while their *in vitro* activity remains intact. This highlights the significance of CCR7 to Treg function through facilitating localization (33, 38, 286, 287).

Native Th1 responses are dependent on CXCR3-mediated signals. Tregs inhibit stable contacts between $CD4^{+}$ T-cells and dendritic cells and can influence the expression of CXCR3 ligands in draining lymph nodes. Early data demonstrated that Treg localization in the lymph node, when suppressing $CD4^{+}$ T-cell responses, was via localization with dendritic cells, rather than the $CD4^{+}$ T-cells, suggesting they act by inhibiting the dendritic cell-$CD4^{+}$ T-cell interaction (193, 194). Further data has suggested, Tregs can also prevent the expression of CXCR3 on effector T-cells, inhibiting their migration and trafficking (288, 289). Tregs can also upregulate T-bet and express CXCR3 in response to INF-γ. These CXCR3 expressing Tregs not only resemble Th1 subsets of effector T-cells, expressing T-bet in addition to FOXP3 but also demonstrate an ability to produce INF-γ and IL-10. Crucially, they are essential to the suppression of Th1-mediated inflammation, and imaging data suggests their function is mediated by colocalization with effector Th1 $CD4^{+}$ and $CD8^{+}$ T-cells in secondary lymphoid organs (36, 290–292).

CCL3 and CCL4 interactions with CCR5 are recognized to be important to $CD4^{+}$ and $CD8^{+}$ T-cell interactions in the lymph node for generating memory $CD8^{+}$ T-cells (293). The production of CCL3 and CCL4 by Tregs has been observed, attracting $CD4^{+}$ and $CD8^{+}$ T-cells via CCR5 expressed on these cells; bringing them into proximity and allowing Tregs to exert their suppressive function. Tregs deficient in CCL3 and CCL4 fail to prevent the progression of experimental autoimmune encephalomyelitis or islet allograft rejection in murine models and Tregs from individuals with type 1 diabetes demonstrate an impaired ability to produce CCL3 and CCL4 (268). Additionally, Tregs can interfere with the priming of $CD8^{+}$ T-cells by modulating the expression of the CCR5 ligands CCL3, CCL4 and CCL5. The deletion of Tregs leads to the stabilization of interactions between dendritic cells and low-avidity T-cells, compromising the responses of high-avidity memory T-cells and memory responses (289, 294).

CXCR5 is key to the function of Tregs in modulating B cell and humoral responses. Chung et al. were able to show Tregs expressing CXCR5 and Bcl-6 localize to germinal centers, with CXCR5 expression being Bcl-6 dependent (295). These follicular Tregs, are absent in the thymus. Their depletion leads to exaggerated germinal center reactions, including germinal center B cells, affinity maturation of antibodies and the differentiation of plasma cells (289, 295). These results were confirmed by work by Linterman et al., which showed follicular Tregs regulate B cell responses, controlling germinal center responses (289, 296–299). Follicular Tregs are hypothesized to exert their effect via various mechanisms, including the production of IL-10, and TGF-β (300–302). Intriguingly, CXCR5 is not necessary for the localization of follicular Tregs, but the transcription factor Bcl-6 is necessary for function and localization (36, 297, 298, 303, 304).

Finally, a $CCR6^{+}$ Treg subset has recently been demonstrated to be significant to thymic Treg development and function. These IL-1R2-positive Tregs express CCR6, but not CCR7, which suggests they recirculate from the periphery with an activated phenotype. These Tregs can quench IL-1, indicating they can maintain tTreg development even in inflammatory conditions (305).

8.2 Tregs in various tissues

Tregs in various tissues demonstrate specificity to site and function. Here we describe selected examples of organ-specific Tregs and the chemokine receptor profiles that facilitate and

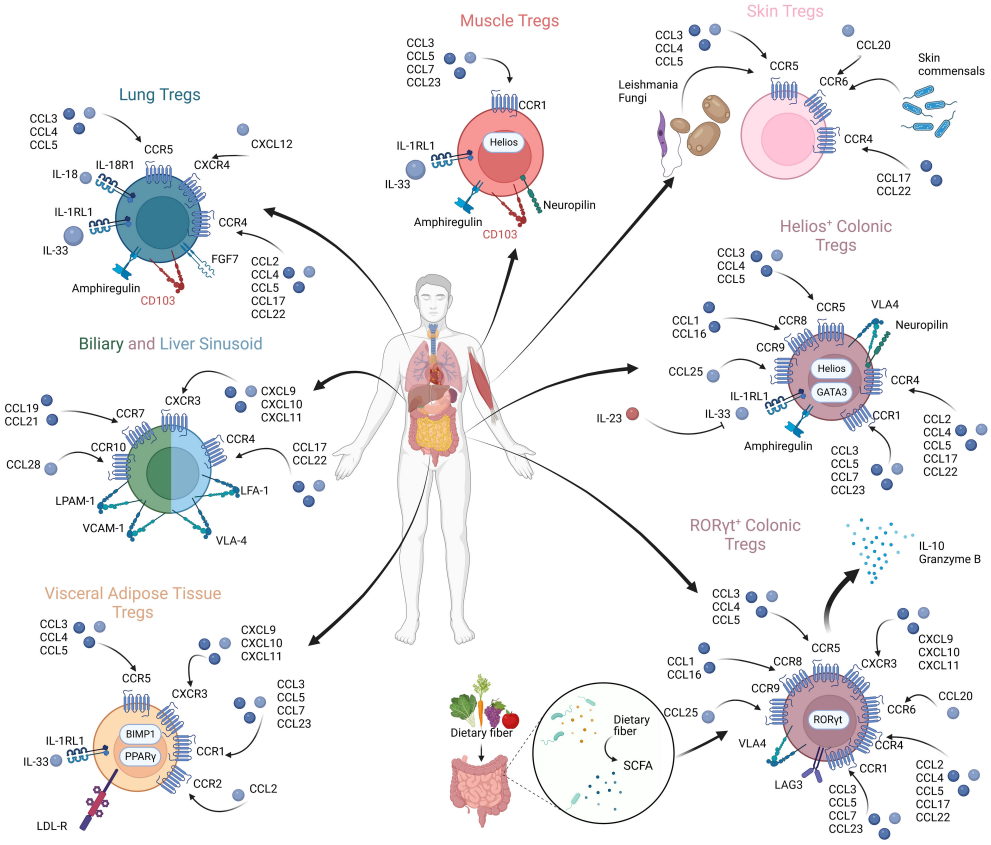

FIGURE 3
Tregs in diverse anatomical compartments exhibiting differential chemokine expressions and chemokine receptor profiles.

define their tissue tropism, allowing immunosuppression within their unique environments (285, 306).

Visceral Adipose Tissue: Tregs in visceral adipose tissue (VAT) show enrichment in CCR1, CCR2, CCR5 and CXCR3, as well as the IL-33 receptor, ST2 (also known as IL-1RL1) (307–309). Tregs in visceral adipose tissue migrate under the influence of the IL-33/ST2 axis, with the majority of IL-33 arising from mesenchymal stromal cells (310, 311). Hydroxyprostaglandin dehydrogenase expressed by VAT Tregs exerts an inhibitory effect on conventional T-cells and inflammation (312). Most interestingly, however, is the limited range and repertoire in Treg TCRs present on VAT Tregs, suggesting they respond to specific antigens found in the tissue of residence (275, 309, 313). This may relate to their function in regulating inflammation within VAT, and thus having a restricted repertoire of antigens which they may need to respond to, as it appears VAT Tregs regulate the inflammatory state and promote insulin sensitivity (314). Knockout of this subset of Tregs leads to impaired function in the insulin receptor and chronic inflammation (314).

Skin Tregs: A significant subset of peripheral blood Tregs appear to express CCR4, but Tregs in certain tissues seem to upregulate the expression of this receptor, with its presence noted in skin, liver, lung and intestinal Tregs (315–317). The loss of CCR4 on murine Tregs impairs Treg accumulation in skin and lung tissues, with complete loss of CCR4 in the Treg cell compartment leading to severe dermatitis, pneumonitis and lymphadenopathy (316).

CCR5 has been shown to be key to the function of many tissue resident Tregs, as well as potentially having a role in persistent infections. In a *Leishmania major* model, CCR5 was essential to the homing of Tregs to *Leishmania* infected dermal sites, promoting infection and parasite survival (318). Similar results have been observed in chronic fungal granulomata, with chronic inflammation mediated by CCR5 expressing Tregs, as pathogens generate CCR5 ligands, leading to Treg recruitment and dampened immune responses (270).

In neonates, CCR6 is crucial to Treg tropism to the skin. Interactions in early life between commensals and CCR6 expressing Tregs induce Treg accumulation at this site, facilitating tolerance to normal skin commensals, mediated by CCL20 and CCR6 interactions (319, 320).

Skeletal muscle and regulatory T-cells: Tregs in muscle are characterized in part by the presence of CCR1 on the surface, as well as ST2 and the key growth factor Amphiregulin (271). These Tregs seem to demonstrate a key role in promoting muscle repair and regeneration (271, 272, 321–323). Tregs in muscle are predominantly recruited from the circulation via the IL-33/ST2 axis, as is seen in VAT Tregs (321). Once in the muscle, CD103 plays a key role in adherence and retention of these Tregs (324). Interestingly, muscle Tregs seem to show a high level of Helios and Neuropilin, suggesting a thymic origin, however, data clearly shows their recruitment from the circulation. Similar to VAT Tregs,

muscle Tregs demonstrate a limited repertoire of TCRs, suggesting specific and limited targets (271). They are recognized to be involved in the repair and regeneration of muscle tissue, having been identified in the inflammatory infiltrate of injured muscle, both in acute and chronic muscle injury, expressing IL-10 where observed (271, 272). Indeed, muscle Tregs were shown to decline with age which likely contributes to defects in muscle regeneration seen in aging (325). Amphiregulin seems to play a crucial role in the regeneration of muscle cells, acting directly on satellite cells, promoting myocyte differentiation *in vitro* and enhancing muscle repair *in vivo* (271, 272, 275, 308, 326).

Lung Tregs: Tregs found in the lung, as discussed above, predominantly demonstrate upregulation in CCR4 (327). Similar to muscle Tregs, Tregs in the lung also appear to have a high expression of amphiregulin. Distinct to these Tregs however is the expression of IL18R1, ST2, CCR5, CXCR4, and KGF with downregulation of CXCR5 upon activation (307, 328). Tregs are recruited to the lung via TGFβ expression by a resident IFNAR1^{hi} TNFR2^{+} conventional dendritic cell 2 (iR2D2) population (329). Tregs play a crucial role in the resolution of lung injury, modulating immune responses and enhancing alveolar epithelial proliferation and tissue repair through the expression of amphiregulin and keratinocyte growth factor (KFG) (330). Inflammatory responses are also dampened by the upregulation of Mmp12, inhibiting neutrophil recruitment. Sik1, meanwhile, has been shown to be downregulated on activation of lung Tregs, leading to an increase in the expression of CD103 (αEβ7) in response, facilitating Treg adhesion and tissue retention (328). Furthermore, Tregs have been highlighted to play a role in asthma. The Notch4 pathway has been found to be upregulated in Tregs in asthmatics. Defective Hippo signaling gives rise to FOXP3 CN2 methylation in Tregs, impairing Treg stability and thus their ability to regulate inflammation in this environment. Thus, the rescue of Tregs in this environment and restabilizing them presents a future therapeutic direction in asthma (273).

Tregs in the gut: The gut provides a dynamic environment, containing a complex interplay of food antigens and resident microbial antigens. As the principal digestive and absorptive site in mammals, an immense range and variety of peptides are present. There is a complex interplay in the gut of various resident and migrating immune cells, from both innate and adaptive systems. Regulating these interactions is essential, and Tregs play a crucial role in orchestrating responses (331). The ablation of colonic Tregs has been demonstrated to lead to aberrant Th1 and Th17 mucosal responses, which are rescued with adoptive transfer (274, 275, 332). Chemokine receptors in gut resident Tregs include CCR1, CCR4, CCR5, CCR8, CCR9 and CXCR3, in addition to the integrin α4β7 (discussed above) (306, 307, 333). This subset of Tregs play a key role in the regulation of complex immune interactions and have been found to perhaps represent two distinct populations, one derived from the thymus and another from peripheral Tregs.

Single-cell transcriptomic data has since shown the colon has populations of tTregs, reflecting those seen in non-lymphoid tissues (expressing GATA-3, neuropillin-1, amphiregulin, ST2 and Helios), and suppressive pTregs (expressing IL-10, Granzyme B, LAG3 and CXCR3) defined by the expression of the transcriptional factor RORγt (306). Interestingly, the microbiome seems to play a crucial role in the recruitment of the latter, being totally absent in germ free mice, and bile acid metabolites impact their function and proliferation (334–337). The two first reports of RORγt Tregs were published together in 2015 (335, 336). Crucially, the data on the function of these cells was divergent, with one group finding that they inhibit Th1/17 responses, while the other demonstrated they respond to Th2 cells. Others have shown that RORγt expression in Tregs may represent a step in the process towards a Th17 Treg phenotype, expressing CCR6 and sharing phenotypic features usually observed in Th17 T-cells (97, 338).

IL-33 has been noted to be present in high quantities in human inflammatory bowel disease, as well as colonic inflammatory lesions. This binds ST2 on Tregs, stimulating responses by enhancing TGF- β1-mediated differentiation and providing signals for the accumulation and maintenance of Tregs in inflamed tissues. Conversely, IL-23, inhibits IL-33 responses. These data suggest that ST2-mediated responses and the balance between IL-33 and IL-23 may contribute to the control of intestinal immune responses (339). Additionally, Blimp-1 has been shown to control the differentiation and function of Tregs, promoting IL-10 generation and ICOS expression in Tregs and suppressing colitis via an IRF4-dependent pathway (340, 341).

Treg/Th17 imbalances are well established to be at play in the pathogenesis of ulcerative colitis. CCR6 Tregs have been shown to be enriched in ulcerative colitis patients and correlated with disease activity. CCR6^{+} Tregs also demonstrated a higher propensity to secrete IL-17A, suggesting that they may play a part in the ongoing colitis (269). RORγt expressing Tregs have been noted to express CCR6 and this may be a marker of Th17 biased Tregs (338).

In addition to the above, a novel chemokine receptor, GPR15, an orphan GPCR which binds to its own ligand (GPR15L) has been shown to play a role in the trafficking of Tregs to barrier tissues, including skin and colon, where it is involved in the suppression of colitis and graft rejection. GPR15 has been shown to have its expression modulated by both gut microbiota and TGF-β, with knockout of this receptor giving rise to colitis, rescued by transfer of Tregs with intact GPR15, suggesting GPR15 has a crucial role to play in mucosal health (36, 342, 343).

Liver Tregs: The liver, is a key organ to gut health and homeostasis. With a dual blood supply, and 75% of this from the portal system, it attracts a high burden of various food and microbial antigens and consequently, a tolerogenic environment has to be maintained. However, appropriate responses must be mounted to remove pathological antigens and particulates, preventing them from entering the systemic circulation (344).

CXCR3 is critical to Treg migration into the liver and liver sinusoids. This chemokine receptor allows for the colocalization of Tregs within the liver, with CCR4 cognate ligands (CCL-17 and -22) allowing for colocalization with infiltrating dendritic cells within inflammatory infiltrates in liver diseases (344, 345). Diseased livers demonstrate elevated expression of CXCR3 on Tregs (258). This adhesion is mediated by CXCL-9, -10 and -11 expression. *In vitro* models have demonstrated the upregulation of this chemokine under stress (346). Contacts made by lymphocytes and sinusoidal

epithelial cells activates integrins, including LFA-1 and VLA-4 (also known as α4β1) on lymphocyte. These integrins engage adhesion molecules, including ICAM, VCAM and VAP-1 on epithelial cells, facilitating Treg transmigration into the hepatic parenchyma (347). This process enables Tregs to access the hepatic sinusoidal environment and may be utilized for clinical applications, as described below (258).

Additionally, CCL28 production in the biliary epithelium recruits CCR10 expressing Tregs. CCL28 is secreted by primary human cholangiocytes in response to LPS, IL-1 and bile acids. The CCL28 - CCR10$^+$ interaction induces adhesion molecule expression, including VCAM-1, and α4β7. Furthermore, CCR10$^+$ Tregs exhibit low expression of CCR7 (indicating a memory phenotype) and high levels of CXCR3 (348).

In inflamed bile ducts, CCL20 is secreted by biliary epithelial cells. In a Th17 inflammatory environment, replicated *in vitro* by a cocktail of IL1β, TNFα, IFNγ and IL-17, CCL20 is secreted by human biliary epithelium and leads to recruitment of CCR6 chemokine receptor expressing effector T-cells to sites of biliary inflammation (97, 349, 350). Additionally, CCR6 is a common Treg marker, associated with Th17 skewing. However, in the liver, CCR6 Tregs are liable to phenotypic instability and conversion to a Th17 inflammatory phenotype. This instability challenges their viablility as a candidate for clinical application or cell therapeutic (258). Additionally, it has been noted that CCR6 expressing Tregs in the colon can mount Th17 responses, regulated by IL-10 production in CCR6 expressing Tregs (97, 351, 352).

By demonstrating tissue selectivity and specificity through chemokine receptors, Treg cell products can be designed for organ-specific cell therapy. Liver diseases such as primary sclerosing cholangitis, primary biliary cirrhosis, autoimmune hepatitis and liver transplant rejection all demonstrate a significant contribution from T-cell immune responses to autoantigens expressed in either the biliary epithelium or hepatocytes (353–365). The proof-of-concept AUTUMN study (Autologous regUlatory T-cells infUsion and tracking in autoiMmuNe hepatitis) employed GMP-manufactured Tregs and found infused Tregs favoured migration to the liver (366).

Tregs in the Bone Marrow: Tregs expressing CXCR4 have been shown to collect in the bone marrow, with loss of CXCR4 impairing accumulation in the bone marrow. The depletion of CXCR4 Tregs has also been shown to lead to an increase in B1 mature B cells, exclusively within the bone marrow, with no observed changes in plasma cells or hematopoietic stem cells and no signs of immune activation elsewhere. However, the loss of these CXCR4 Tregs does lead to an increase in total serum IgM levels and a highly specific increase in IgM autoantibodies (367).

8.3 Chemokine receptors in cancer

Tregs are enriched in the tumour microenvironment, and their enrichment has been correlated with worse outcomes (368). Tregs in the tumour microenvironment supress host immune responses, subverting immune destruction and thus supporting tumor growth (369). Consequently, Tregs have become a focus of cancer immunotherapy (370). However, specifically targeting cancer facilitating Tregs and Tregs in the tumour microenvironment remains a barrier to effective cancer immunotherapy with limited offsite side effects (371, 372).

CCR4 is predominantly expressed in tumor resident Tregs, enabling tumor immune evasion, and is thus regarded as a potential candidate target for cancer immunotherapy (71, 373, 374). CCR8's more recent identification as a chemokine receptor specifically upregulated in cancer Tregs and it's correlation with poor outcomes in patients has garnered significant interest (36, 375, 376). This cytokine receptor has been shown to be upregulated in colorectal, non-small cell lung, breast, and other cancers. Additionally, some data has shown CCR8 to be upregulated upon Treg activation, with autocrine production of CCR8's ligand CCL1 (377). Data on targeting CCR8 in cancer thus far, however, has been conflicting, but some preclinical models have shown promise (378–381).

Nevertheless, both CCR4 and CCR8 appear to be expressed under the control of GATA3 and IRF4, both of which are canonical Th2 transcription factors (36). The expression of these transcription factors on activated Tregs hints at a Th2 skewed Treg population and suggests that CCR4 and CCR8 may be chemokines specific to Th2 Treg function (382–384). GATA-3, meanwhile, has been shown to be essential to Treg function, binding to the CNS2 in Foxp3 promoting Foxp3; with loss of GATA-3 leading to a 50% reduction in FOXP3 mRNA transcripts compared to baseline, with the same seen in other Tregs signature genes (CD25, CTLA-4, GITR). Furthermore, GATA-3 deficient Foxp3 Tregs acquire Th17 cytokine expression profiles (383, 385).

CCR6 has been shown to be a target of certain cancer-derived factors, promoting Treg migration. Eomesodermin has been shown to be generated by oesophageal adenocarcinoma, and this has been shown to drive CCL20 secretion, which binds to CCR6 on Tregs driving Treg chemotaxis and residency intratumorally, promoting cancer growth (386).

8.4 Chemokine receptors in graft-versus-host disease

GVHD is a complication affecting every second stem cell transplanted patient. Both acute and chronic GvHD are characterised by a systemic inflammation of recipient tissue caused by donor cells in the graft. Severe treatment refractory GvHD can often be lethal. Polyclonal Tregs have successfully been used in named patient programs and clinical trials by us (Trzonkowski; Fuchs(Theil)) and others, both to prevent and to treat GVHD (387–393).

In a murine model of GVHD, the knock-out of CCR5 impaired the migration of donor Tregs to GVHD target organs, leading to lethal outcomes (394). Additionally, CCR8 in GVHD has been shown to promote Treg maintenance, by allowing tolerogenic interactions with donor CD11c+ antigen presenters. In a murine model of islet transplantation, CCR5 along with CCR2, CCR4 and P- and E-selectins were essential to the movement of Tregs from blood into pancreatic islet allografts. Subsequent migration to

lymph nodes was found to be CCR2, CCR4 and CCR7 dependent, from which they could inhibit effector T-cell responses in both the allograft and lymph node (395).

9 Genetic engineering to enhance Treg function

Deep understanding of Treg biology, mechanisms of immunosuppression, as well as identification of chemokine receptor patterns responsible for tissue tropism are cornerstones for the generation of genetically modified cells with improved function and enhanced infiltration of the target tissue. Polyclonal autologous Tregs have demonstrated efficacy in several trials, but accumulating evidence from preclinical models demonstrate increased potency of antigen-targeted approaches (1) (388, 390, 396–399). Recent success and FDA/EMA approval of chimeric antigen receptor (CAR)-T-cells in hematological cancers has caused a paradigm shift (400), with increased interest in CAR-Tregs. For example the technology enables generation of Tregs that will precisely target HLA mismatches in transplantation or antigens within inflamed tissues, facilitating direct local suppressive effects. CAR constructs in general are built from: 1) an extracellular ligand binding domain which consists of the antigen-specific variable fragment of heavy and light antibody chains (single-chain variable fragment; scFv), 2) hinge region (providing scFv flexibility), 3) transmemberane domain (that anchors the receptor in plasmalemma) and finally 3) an intracellular costimulatory domain derived from T-cells (401, 402). Until now five generations of CARs have been developed and tested with the main difference in the intracellular domain. The first-generation CARs comprised CD3ζ, a part of the T-cell receptor-CD3 complex, while second-generation CARs combine CD3ζ with costimulatory molecules such as CD28, CD137, CD27 or CD134 delivering a second signal and were the most extensively studied approach. The third-generation CARs comprise of two costimulatory molecules (403–405), fourth generation CARs co-express cytokine or chemokine genes, While the fifth generation receptors contain intracellular domain of a cytokine receptor (e.g., IL2RB chain) that interacts with STAT3 (406–409). In case of genetic Treg modifications, mostly 2nd generation CARs were tested. The Levings´group conducted a comprehensive study exploring the Treg immunosuppressive capacity and stability after incorporation of various costimulatory domains into the CAR (including CD28, ICOS, CTLA4, PD1, GITR, OX40, CD137, and TNFR2) (410). Interestingy, in contrast to anti-tumor conventional CAR-T cells, Tregs with CD28-encoding CAR exhibited superior in vitro and in vivo performance in terms of proliferation, suppression, and delaying GvHD symptoms. In addition, both CD137- and TNFR2-CARs were found to negatively affect Treg function and stability, leading to FOXP3 locus methylation, decreased Helios expression, and reduced suppressive function in vitro and in vivo. Interesting the group published very recently, that Tregs expressing CARs encoding CD28, ICOS, PD1, and GITR, but not 4-1BB or OX40, all extended skin allograft survival in an immunocompetent transplant model (411). With other studies (412, 413) consistently reporting adverse consequences of using CD137 in Tregs, one may conclude that this co-stimulatory domain is suboptimal for CAR-Tregs. However, in the case of flexible modular chimeric antigen receptor technology called universal CAR (UniCAR-Tregs), both CD137 and CD28 costimulation induced robust suppressive capabilities. Nevertheless, due to higher background activation of Treg in case of CD28, UniCARs featuring a CD137-CD3ζ signaling domain were discussed as the preferred constructs for the clinical application of redirected Tregs (414). An intriguing avenue for exploration involves combining CD28 and CD137 domains to potentially optimize CAR-Treg suppression, as reviewed by Zhang, Qunfang et al. (415).

Noteworthy, IL-10 secretion was successfully induced into 4th generation CAR-Tregs, enhancing suppressive function (416). However, no reports on fifth- generation CAR-Tregs have been available yet and their specific effects on CAR-Tregs needs further investigation. The state of knowledge on CAR-Treg generation is summarized in a review by M. Levings, 2020 (417). Further innovative adaptations of CAR designs have emerged with the potential to enhance the durability, stability, proliferative capacity, and function of CAR-Tregs. Additionally, novel CAR-Tregs specific for E.coli-derived flagellin have been designed and introduced into humanized murine models. These FliC-CAR-Tregs were directed to the colon (which confirmed improved homing abilities) and were characterized by higher expression of PD-1, highlighting higher immunosuppressive capacity and potential for application in inflammatory bowel diseases (418). Adoptive Treg therapy is limited by the plasticity of Tregs, potentially transforming into conventional T-cells. To overcome this challenge, additional genetic engineering has been suggested to enhance Treg stability and robustness. Examples include engineered epigenetic and post-transcriptional changes in FOXP3 and metabolic stabilization by enhancing CD39 and CD73 expression. Elevating IL-10, TGFβ, IL-34, IL-35, and FGL-2 can boost Treg function, while suicide genes help manage Treg adverse effects. This idea of the next generation of Super-Tregs with increased function, stability, redirected specificity and survival is summarized by Amini et al. (419).

Engineered antigen-specificity has demonstrated improved responses in mouse models, emphasizing utility (420, 421). In mouse models, researchers demonstrated that lentiviral transduction did not alter the Treg phenotype and HLA-A2 CAR-Tregs were effective in suppression. CAR-Tregs produced small amounts of IFN-ɣ, but more importantly, secreted the highest amounts of IL-10. Finally, transduced cells migrated more rapidly through HLA-A2+ HUVECs and contributed to the survival of human skin grafts (416).

Currently, CAR-Tregs remain under investigation (422), and two clinical trials focused on transplantation are registered on clinicaltrials.gov. Sangamo Therapeutics in 2021 initiated the first trial using CAR-Tregs (NCT04817774), infusing TX200-TR101 into HLA-A02-negative patients awaiting kidney transplants from HLA-A02-positive donors. Encouraging preclinical studies demonstrated specific activation and potent allogeneic Tconv suppression by TX200-TR101, both *in vitro* and *vivo*. No side effects have been reported underscoring its potential as a safe and effective therapeutic alternative (423–425). Quell Therapeutics's

QEL-001, similar to TX200-TR101, utilizes an HLA-A2-specific CAR to abrogate immunosuppression (426, 427)Tregs, and the LIBERATE clinical trial (NCT05234190) is currently enrolling patients who have received HLA A2-mismatched liver transplants 12 months to 5 years prior the time of enrolment.

Sonoma Biotechnologies's pipeline includes SBT-77-7101, a CAR-Treg product specific to citrullinated vimentin (CV), a known antigen present in the synovial fluid (SF) of Rheumatoid arthritis (RA) patients (428). Preclinical studies (429–432) have shown promise in using Treg therapy for Systemic lupus erythematosus (SLE), demonstrating the as-yet unrealized potential benefit of these novel technologies.

Additionally, an inventive way of using Tregs in autoimmune disease treatment is planning to be introduced into clinical trials on Multiple sclerosis (MS) and type 1 diabetes. The company "Abata Therapeutics" started to engineer Tregs expressing TCRs recognizing tissue-restricted antigens. Similar to the CAR concept, their TCR-based targeted methods reduce systemic suppressive response limiting it to the specific tissue to which autoimmune response is directed. The company PolTREG develops TCR-engineered Tregs in type 1 diabetes. Interestingly, this company works in neuroinflammatory conditions, such as MS and amyotrophic lateral sclerosis, using a CAR-based concept in which CAR-Tregs are designed to strengthen the blood-brain barrier. As the engineered Tregs landscape expands, continued investigation will unveil the most effective strategies to unlock the full potential of engineered Tregs.

10 Concluding remarks

Tregs constitute a minor fraction of the broader population of $CD4^+$ T-cells. The lack of an exclusive Treg marker together with phenotypical similarities to activated CD4+ effector cells present challenges in the context of cell therapy. However, a variety of markers, often applied in combinatorial approaches identifies functional suppressive Tregs and distinct subsets, and contributed to an enhanced understanding of biological processes. As our knowledge improves and we enter the era of engineered Tregs, significant strides addressing pathological immune-mediated processes can be anticipated. Learnings from ongoing clinical trials, advancement in methods to determine Treg function, together with means to identify patients who are most likely to benefit from Treg therapy, will determine the fate of Treg cell therapy.

Author contributions

SS: Conceptualization, Writing – original draft, Writing – review & editing, Validation. KK: Writing – original draft, Writing – review & editing, Validation. MG: Writing – original draft, Validation. YH: Writing – review & editing, Validation. DI-G: Writing – original draft, Validation. MP-M: Writing – original draft, Validation. JS: Writing – original draft, Validation. MT: Writing – original draft, Validation. JM: Writing – review & editing, Validation. KL: Writing – review & editing, Validation. NM-T: Supervision, Writing – review & editing, Validation. PT: Supervision, Writing – review & editing, Validation. YO: Supervision, Writing – review & editing. AF: Conceptualization, Funding acquisition, Supervision, Writing – review & editing, Validation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Funding for publication was received from "The Saxon State and University Library (SLUB), Dresden". SS and YH are funded by the German Federal Ministry of Education and Research under the funding code 03ZU1111AA as part of the Cluster4Future, SaxoCell. JM, KL, and AF are funded by Mildred Scheel Early Career Center (MSNZ), German Cancer Aid (Deutsche Krebshilfe, DKH). KK and YO are funded by Sir Jules Thorn Biomedical Research Charity, London. KK is funded by the National Institute for Health and Care Research (NIHR). NM-T is supported by the project "International Centre for Cancer Vaccine Science" that is carried out within the International Research Agendas Programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund. NM-T is supported by the project "International Centre for Cancer Vaccine Science" that is carried out within the International Research Agendas Programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund (MAB/2017/3).

Acknowledgments

We would like to express our sincere appreciation to all the contributors and reviewers for their valuable insights and contributions to this review.

Conflict of interest

The authors MG, Dl, MP-M, JS, MT and PT are employed by Poltreg S.A.

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.

The author(s) PT and NM-T declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 25 January 2024
DOI 10.3389/fimmu.2024.1292158

OPEN ACCESS

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RECEIVED 11 September 2023
ACCEPTED 08 January 2024
PUBLISHED 25 January 2024

CITATION
Liu Z, Baines KJ, Niessen NM, Heer MK, Clark D, Bishop GA and Trevillian PR (2024) Characterizing Foxp3$^+$ and Foxp3$^-$ T cells in the homeostatic state and after allo-activation: resting CD4$^+$Foxp3$^+$ Tregs have molecular characteristics of activated T cells. *Front. Immunol.* 15:1292158. doi: 10.3389/fimmu.2024.1292158

Characterizing Foxp3$^+$ and Foxp3$^-$ T cells in the homeostatic state and after allo-activation: resting CD4$^+$Foxp3$^+$ Tregs have molecular characteristics of activated T cells

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Due to the intracellular expression of Foxp3 it is impossible to purify viable Foxp3$^+$ cells on the basis of Foxp3 staining. Consequently CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) in mice have mostly been characterized using CD4$^+$CD25$^+$ T cells or GFP-Foxp3 reporter T cells. However, these two populations cannot faithfully represent Tregs as the expression of CD25 and Foxp3 does not completely overlap and GFP$^+$Foxp3$^+$ reporter T cells have been reported to be functionally altered. The aim of this study was to characterize normal Tregs without separating Foxp3$^+$ and Foxp3$^-$ cells for the expression of the main functional molecules and proliferation behaviors by flow cytometry and to examine their gene expression characteristics through differential gene expression. Our data showed that the expressions of Foxp3, CD25, CTLA-4 (both intracellular and cell surface) and PD-1 was mostly confined to CD4$^+$ T cells and the expression of Foxp3 did not completely overlap with the expression of CD25, CTLA-4 or PD-1. Despite higher levels of expression of the T cell inhibitory molecules CTLA-4 and PD-1, Tregs maintained higher levels of Ki-67 expression in the homeostatic state and had greater proliferation *in vivo* after allo-activation than Tconv. Differential gene expression analysis revealed that resting Tregs exhibited immune activation markers characteristic of activated Tconv. This is consistent with the flow data that the T cell activation markers CD25, CTLA-4, PD-1, and Ki-67 were much more strongly expressed by Tregs than Tconv in the homeostatic state.

KEYWORDS

regulatory T cells, Foxp3, CTLA-4, PD-1, T cell proliferation, differential gene expression

1 Introduction

CD25 (1) and Foxp3 (2, 3) are the two most important biomarkers for identification of Tregs, with the former often used for Treg purification and the latter as the definitive Treg lineage marker (4). Here, we defined $CD4^+Foxp3^+$ T cells and $CD4^+Foxp3^-$ T cells as Tregs and Tconv respectively. As the α sub-unit of the IL-2 receptor, CD25 is uniquely expressed by Tregs but not Tconv in the homeostatic state and confers higher affinity of Tregs for IL-2 than Tconv (5–8). This is the foundation of low-level IL-2 treatment to specifically induce Treg but not Tconv expansion. There is, however, incomplete overlapping of Foxp3 and CD25 expression reported using transgenic green fluorescence protein (GFP) reporter mice (4), which indicated that CD25 is not specifically expressed on Tregs. As the GFP^+Foxp3^+ T cells were reported to be functionally altered due to transgenic manipulation (9, 10), we re-examined CD25 and Foxp3 expression and quantified the degree of their co-expression in normal mice of the same background as GFP mice. In addition to CD25 and Foxp3, the co-inhibitory molecule CTLA-4 was shown to be constitutively expressed in resting Tregs (11) and to control Treg-mediated immunosuppression (12). CTLA-4 is unusual in that although it is CTLA-4 on the cell surface that executes its function, most CTLA-4 molecules are located intracellularly. We were interested to examine the degree of co-expression between Foxp3 and CTLA-4 (intracellular and surface) and the correlation between intracellular and surface CTLA-4 expression. At the same time, the co-expression of Foxp3 and another co-inhibitory molecule PD-1 was also characterized. As CTLA-4 and PD-1 were mostly confined to the $CD4^+$ T cell subset, their expressions by Tregs and Tconv in the homeostatic state and after skin transplantation were examined.

As it is impossible to purify viable $Foxp3^+$ Tregs from normal animals, the proliferative behavior of Tregs is mostly assessed using $CD4^+CD25^+$ T cells which are different from $CD4^+Foxp3^+$ T cells because of incomplete overlap between the expression of CD25 and Foxp3. In addition, the purified $CD4^+Foxp3^+$ T cells from the transgenic GFP reporter mice were reported to be functionally altered (9, 10), and may not represent the actual proliferation of wild-type Tregs. Hence, we decided to examine the proliferation of Tregs and Tconv simultaneously without separating $Foxp3^+$ and $Foxp3^-$ cells in four scenarios: *in vitro* expansion of $CD4^+$ T cells stimulated by allo-antigens assessed by carboxyfluorescein succinimidyl ester (CFSE); *in vivo* expansion of adoptively transferred $CD4^+$ T cells assessed by CFSE; basally proliferating Tregs and Tconv in the homeostatic state assessed by Ki-67; *in vivo* expansion of endogenous Tregs and Tconv in skin transplant recipients assessed by Ki-67. Flow cytometric analysis of CFSE is one of the best ways to assess T cell proliferation, however, it is impossible to label T cells in normal mice with CFSE. Consequently, Ki-67 which is reported to have similar sensitivity as CFSE to detect antigen specific or allo-activated T cells (13) was used to quantify the proliferating cells in the third and fourth scenarios.

Foxp3, CD25, CTLA-4, and PD-1 are not only expressed by resting or naïve Tregs, but also are activation markers for Tconv (reviewed in (14)), which indicates that naïve Tregs share some molecular characteristics of activated Tconv. Hence, a more extensive examination of differential gene expression in resting Tregs and activated Tconv was compared with resting Tconv using the nCounter® Immune Exhaustion Panel containing 785 genes covering immune activation and immunosuppression themes.

2 Materials and methods

2.1 Mice

C57BL/6 (CD45.2, H^{-2b}) mice were purchased from the Central Animal House of the University of Newcastle, Australia. B6D2F1 (CD45.2, H^{-2bd}) and congenic B6ly5.1 (CD45.1, H^{-2b}) mice were purchased from the Animal Resource Centre in Western Australia. The only difference between B6ly5.1 mice and C57BL/6 mice are the CD45 isoform, with the former CD45.2 and the latter CD45.1. Monomeric red fluorescent protein (mRFP) mice (CD45.1, H^{-2b}) were supplied by Associate Professor Alexandra Sharland from the University of Sydney. The mRFP mice (also known as Foxp3-IRES-mRFP mice) contained a knock-in gene faithfully co-expressing with Foxp3 in lymphocytes. All mice were male and from 6 to 12 weeks old. The mice were housed in Hunter Medical Research Institute. The ethics for use of animals was approved by the Animal Care and Ethics Committee of the University of Newcastle (A-2019-920).

2.2 Cell preparation

Cell isolation from lymph nodes and spleen were performed separately, but the same procedure was used for both. When preparing cells used for cell culture or adoptive transfer, the procedures were performed in a sterile class II biosafety cabinet. All the operations except centrifugation were performed on ice. The centrifuge was set at 450×g for 5 minutes at 4°C. The spleen or lymph nodes were transferred to a 70 μm strainer which was placed in a 100 mm petri-dish. The lymph nodes or spleen were then mashed with a 5ml syringe plunger in the 70μm strainer with cold phosphate buffered saline (PBS). After completion of the mashing, the cells were filtered into a 50 ml centrifuge tube through the 70μm strainer. The red cells were then lysed in lysis buffer for 5 minutes at room temperature. After washing, the cells were re-filtered through a 30-μm filter to remove cell clumps and re-suspended in PBS for cell counting.

2.3 Magnetic cell isolation

$CD4^+$ T cells were isolated from pooled spleen and lymph node cells. The isolation was performed through magnetic negative selection using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, #130-104-454) as per manufacturer's instructions. Procedures were performed in a sterile class II biosafety cabinet. The purity of isolated $CD4^+$ T cells was over 90% assessed by flow cytometry.

The isolated $CD4^+$ cells from B6ly5.1 mice were labelled with CFSE for *in vitro* cell culture and adoptive transfer experiments. The isolated $CD4^+$ cells from mRFP mice were used for NanoString differential gene expression analysis.

2.4 CFSE labelling

All procedures minimised exposure to light. CFSE was diluted by adding 90µl DMSO to one vial of CFSE to make a 10 mM solution, which was further diluted 100 times to make a working solution before use. The cells were suspended in a concentration of 2×10^7/ml in 2% FCS Dulbecco's phosphate-buffered saline (DPBS). 50µl of diluted working concentration CFSE per 1ml of the suspended cells achieved a final concentration of 5 µM and the cells were incubated at room temperature for 5 minutes. The CFSE staining was quenched using 9 ml 10% FCS DPBS for 2 minutes. The CFSE stained cells were washed twice using 2% FCS DPBS and suspended in a suitable concentration for downstream work.

2.5 *In vitro* $CD4^+$ T cell stimulation

$CD4^+$T cells from the lymph nodes and spleen of each B6ly5.1 mouse were pooled and labelled with CFSE as responders and the irradiated splenocytes (15 Gray) from B6D2F1 mice as stimulators. The *in vitro* culture was performed as described in reference (15) for 3 days.

2.6 *In vivo* $CD4^+$ T cell stimulation by adoptive T cell transfer

The $CD4^+$ T cells from lymph nodes and spleens of all donor B6ly5.1 mice were pooled together before CFSE labelling. The cells were resuspended in sterile 0.9% injection saline and adoptively transferred to recipient B6D2F1 mice on a one-donor-to two-recipients basis. The injection volume for each mouse was 400 µl. After sufficient anaesthesia, the cells were injected to the recipient mice through the penile vein using a gauge 25 injection needle under the microscope with the dimmest microscopic light possible. After the injection was complete, the penis tip was gently pressed and pushed back into the prepuce using a sterile cotton bud. The mouse normally recovered in 2-5 minutes and then was transferred to a clean holding cage for further monitoring. The cells from lymph nodes and spleens were isolated and analysed separately at day 3.

2.7 Skin transplantation

C57BL/6 (H-2^b) or mRFP (H-2^b) mice were graft recipients and B6D2F1(H-2^{bd}) mice were skin graft donors. 1×1 cm full-thickness tail skin was grafted on the back of the recipient mice. The skin graft was secured with medical grade glue and wrapped using adhesive bandage. The bandage was removed 7 days after transplantation.

2.8 Antibody staining for flow cytometric analysis

The antibodies and other reagents for flow cytometry work are listed in Table 1.

2.5 million cells were stained in a volume of 100µl in a FACS tube on ice. After Fc blocking, the cells were incubated with fluorochrome conjugated antibodies for cell surface molecules for 30 minutes with the addition of Brilliant Stain Buffer to make 100µl volume. After complete washing, the cells were incubated with the viability dye for another 30 minutes. The fluorochrome conjugated antibodies for intracellular molecules were stained for 30 minutes at room temperature after fixation and permeabilization using the Foxp3 Buffer Set as per manufacturer's instructions.

All data acquisition was performed on the same BD LSR Fortessa X-20 cytometer and analysed using FlowJo 10.8.1. The expression level was assessed by median fluorescence intensity (MFI). The gating strategy for Foxp3, CD25, intracellular CTLA-4, surface CTLA-4, PD-1, and Ki-67 in skin transplant experiment is shown in Supplementary Figure 1. The gating strategies to gate Tregs and Tconv in *in vivo* (A and B) and *in vitro* (C and D) expansion is shown in Supplementary Figure 2.

2.9 Flow Cytometry Cell Sorting (FACS)

$CD4^+$ T cells that had been isolated by magnetic cell isolation from mRFP reporter mice were then stained with viability dye for cell sorting for $mRFP^+Foxp3^+$ (Tregs) and $mRFP^-Foxp3^-$ (Tconv) cells by FACSAria III sorter. The mRFP was assessed in the PE channel. The gating strategy for Tregs and Tconv sorting are shown in Supplementary Figures 2E, F. The generated FACS data was re-analyzed in Flowjo and the gating strategy for Tregs and Tconv is shown in Supplementary Figures 2G, H.

2.10 Differential gene expression

The FACS sorted Tregs and Tconv were lysed in QIAGEN Buffer RLT Plus (# 1053393) with β-ME and stored at -80°C until RNA extraction. RNA was extracted using QIAGEN RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Digital gene expression profiles were analysed using the nCounter Mouse Immune Exhaustion Panel (see gene list document and panel description in https://nanostring.com/products/ncounter-assays-

TABLE 1 Antibodies and viability dyes used in flow cytometry.

Viability or antibodies	Clone	Fluorophore	Company	Catalog	Optimal dilution
CD45.1	A20	BV605	BD Biosciences	747743	1/200
CD3e	500A2	R718	BD Biosciences	567303	1/200
CD3e	145-2C11	FITC	BD Biosciences	553061	1/200
CD4	GK1.5	BV786	BD Biosciences	563331	1/800
CD8a	53-6.7	APC-H7	BD Biosciences	560182	1/100
CD25	PC61	PE-cy7	BD Biosciences	552880	1/200
Foxp3	FJK-16S	PE	eBioscience	12-5773-8 2	1/200
CD152 (CTLA-4)	UC10-4F10-11	APC	BD Biosciences	564331	1/200
CD279 (PD-1)	RMP1-30	BV421	BD Biosciences	564071	1/200
Ki-67	B56	BUV395	BD Biosciences	564071	5μ/reaction
Live/dead	n/a	Fixable Viability Stain 510	BD Biosciences	564406	1/1000
Live/dead	n/a	LIVE/DEAD™ Fixable Near IR (780) Viability Kit (NIR)	eBioscience	L34994	1/1000

n/a, not applicable.

panels/immunology/immune-exhaustion/), as per manufacturer's instructions (NanoString Technologies).

The data was analysed using the Rosalind platform (https://www.rosalind.bio/). Differential gene expressions between T cell groups were determined as a p value<0.05 adjusted for false discovery rate using the Benjamini-Hochberg method, and a fold change (FC) of 1.5. The data was displayed as $\log_2$ FC [whereby ±1.5-FC difference is equivalent ±0.58 $\log_2$ FC].

2.11 Statistical analysis

The statistical analysis of the data in this project was performed using GraphPad Prism 9. Paired two-tailed t test and one-way ANOVA were used when comparing 2 and over 2 parameters, respectively, of samples from the same mouse. Likewise, unpaired two-tailed paired t test and one-way ANOVA were used when comparing 2 and over 2 parameters, respectively, of samples from different groups of mice. The data was shown in the figures as mean ± standard deviation (SD). The Log-rank (Mantel-Cox) test was used for graft survival analysis. It was considered significantly different if $p < 0.05$. For the range of values from $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ were shown as ns, *, **, *** and **** respectively. If the p value was near 0.05, the exact p value was shown. The data was shown in the figures as Mean ± SD. The differentially expressed genes in the differential gene expression experiments were screened using adjusted $p < 0.05$.

3 Results

3.1 The expressions of Foxp3, CD25, CTLA-4 and PD-1 is mostly confined to $CD4^+$ T cells with their highest expression in CD4/CD8 double positive T cells in the normal homeostatic state

As shown in Supplementary Figure 1G, the frequency of CD4/CD8 double negative (DN) T cells in total T cells was low (less than 6%) and the frequency of DP T cells was much lower (less than 0.5% of total T cells). This is a contrast to $CD4^+$ T cells ranging from 48.3% to 54.6% and $CD8^+$ T cells ranging from 40.2% to 45.7%.

Consistent with the published data from GFP mice (4), our results from normal C57BL/6 mice showed that approximately 97% of $Foxp3^+$ T cells were confined to the $CD4^+$ compartment (Figure 1A-1, A-2). It is important to point out that the distribution of $Foxp3^+$ T cells into the $CD8^+$ and DP compartments was at comparable levels, although the former was about 100 times the latter in cell number. Similar to $Foxp3^+$ cells, the vast majority of $CD25^+$, intracellular CTLA-4^+, surface CTLA-4^+ and PD-1^+ T cells were also limited to $CD4^+$ T cells (Figure 1B-1–E-1, B-2–E-2).

The highest frequencies of $Foxp3^+$, $CD25^+$, intracellular CTLA-4^+, surface CTLA-4^+, and PD-1^+ T cells were always in DP T cell

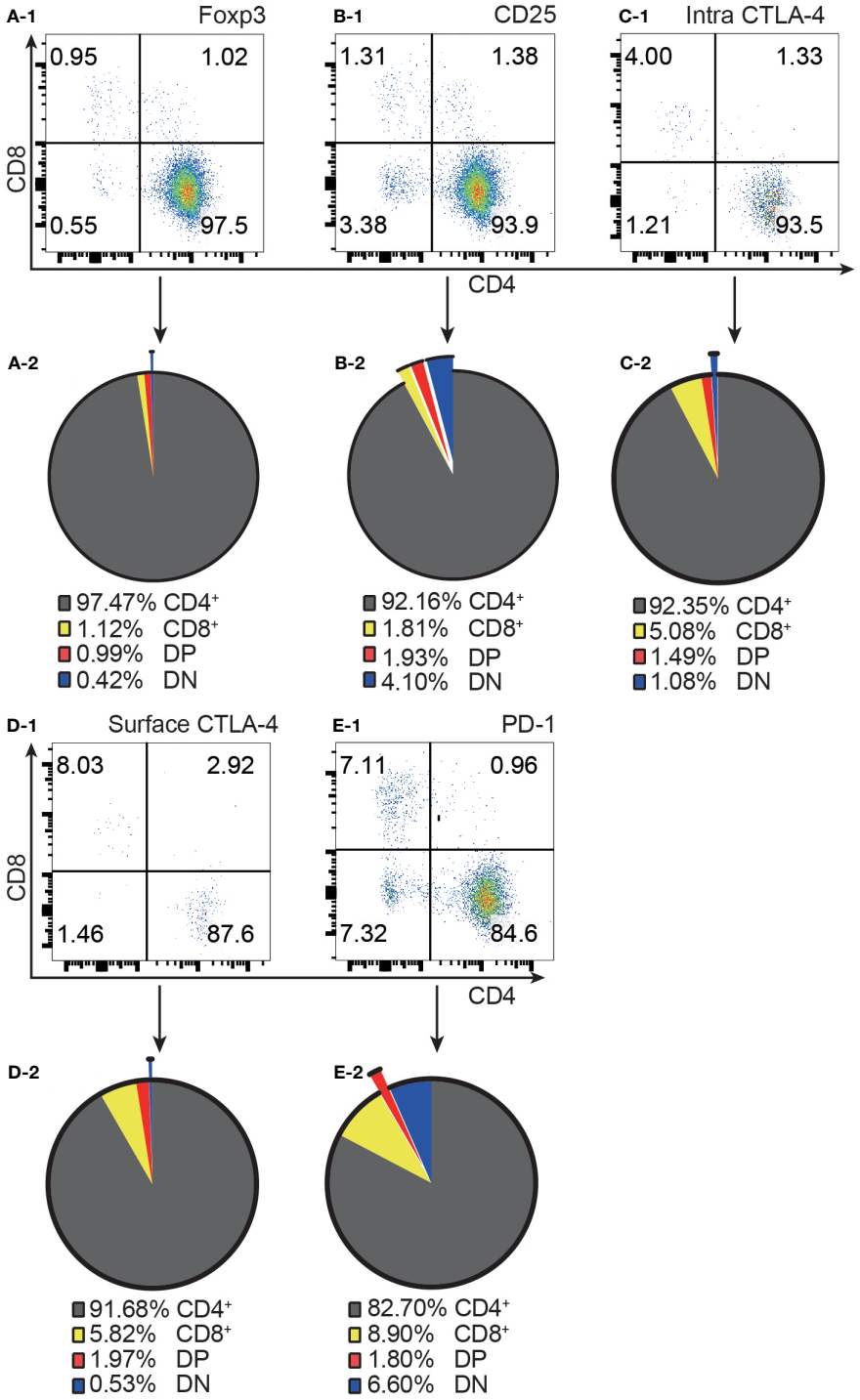

FIGURE 1
The distribution of Foxp3$^+$, CD25$^+$, intracellular CTLA-4$^+$, surface CTLA-4$^+$, and PD-1$^+$ T cells in the four T cell subsets. **(A-1, B-1, C-1, D-1, E-1)** show representative flow cytometry data of the distribution of Foxp3$^+$ T cells, CD25$^+$ T cells, intracellular CTLA-4$^+$ T cells, surface CTLA-4$^+$ T cells, and PD-1$^+$ T cells respectively in the four T cell subsets from normal C57BL/6 lymph nodes. The quadruple gates were set on CD3 positive Foxp3$^+$ cells, CD25$^+$ cells, intracellular CTLA-4$^+$ cells, surface CTLA-4$^+$ cells, and PD-1$^+$ cells respectively. The pie charts of **(A-2, B-2, C-2, D-2, E-2)** show average percentage of the distribution of Foxp3$^+$ T cells, CD25$^+$ T cells, intracellular CTLA-4$^+$ T cells, surface CTLA-4$^+$ T cells, and PD-1$^+$ T cells respectively in the four T cell subsets, (n=7). The data in spleen shows the same pattern.

subset and their frequencies were slightly lower in CD4$^+$ T cells and always lowest in CD8$^+$ T cells (Figure 2A-1–D-1). The expression patterns of Foxp3 and CTLA-4 were the same in that their positive frequencies were higher in DP and CD4$^+$ T cells than in DN and CD8$^+$ T cells (Figures 2A-1, C-1). The expression patterns of CD25 and PD-1 were the same in that their positive frequencies were higher in DP, CD4$^+$ and DN T cells than in CD8$^+$ T cells (Figure 2B-1, D-1). The frequencies of Foxp3$^+$, CD25$^+$, intracellular CTLA-4$^+$,

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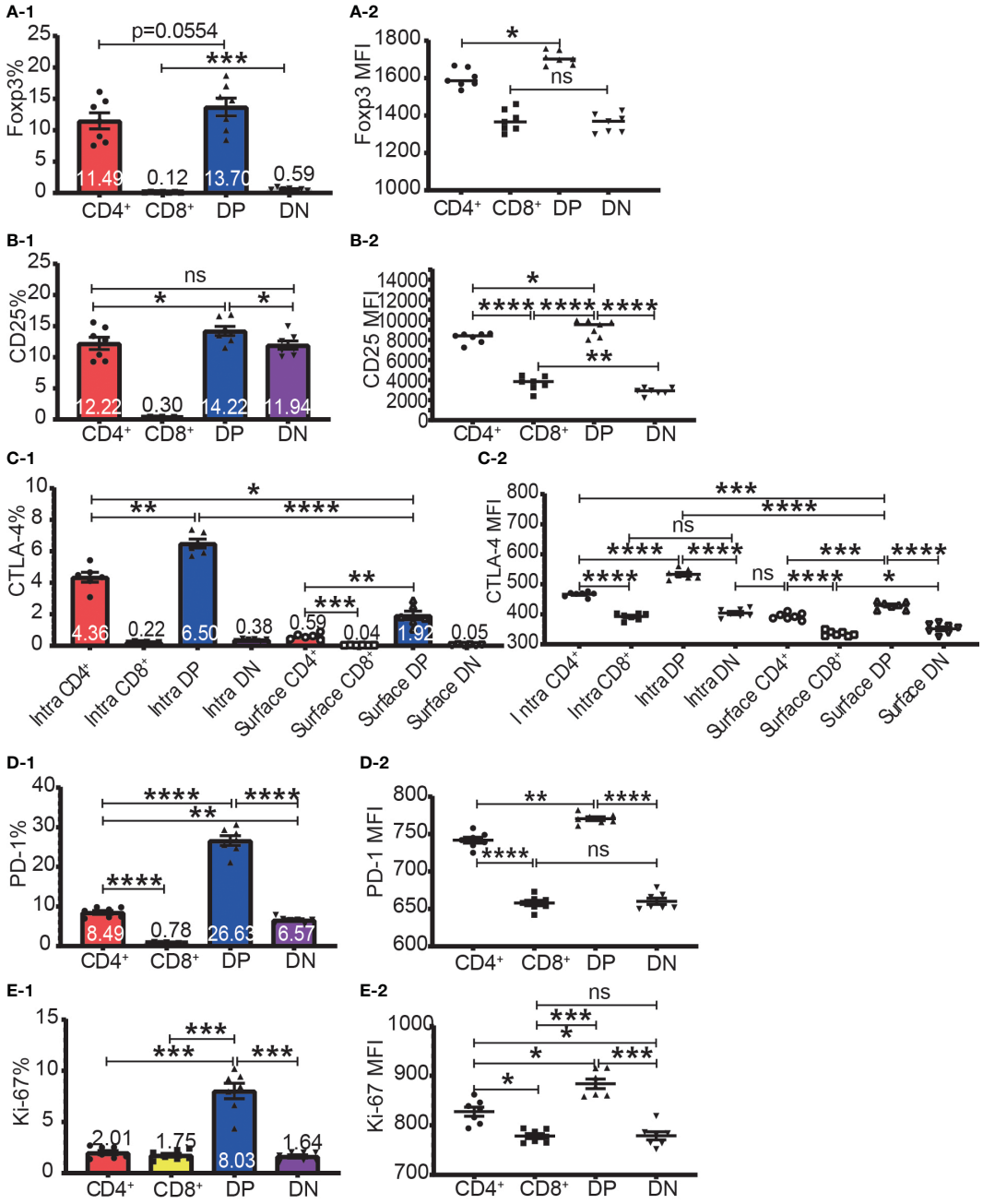

FIGURE 2
The expression of Foxp3, CD25, CTLA-4, PD-1, and Ki-67 in the four T cell subsets. The percentages of Foxp3$^+$ cells, CD25$^+$ cells, intracellular CTLA-4$^+$ cells, surface CTLA-4$^+$ cells, PD-1$^+$ cells, and Ki-67$^+$ cells in the four T cell subsets are shown in **(A-1, B-1, C-1, D-1, E-1)** respectively. The expression levels of Foxp3, CD25, intracellular CTLA-4 and surface, PD-1, and Ki-67 in the four T cell subsets are shown in **(A-2, B-2, C-2, D-2, E-2)** respectively. The data demonstrated is from lymph nodes of normal C57BL/6 mice and is shown as Mean ± SD (n = 7). Multiple-group comparison was performed using paired One-way ANOVA. For the range of values from $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ were shown as *, **, *** and **** respectively. Ns, not significant.

surface CTLA-4$^+$, and PD-1$^+$ cells in CD8$^+$ T cell subsets were all very low. For example, the Foxp3$^+$ cell percentage in CD8$^+$ T cells was less than 0.25% and as low as 0.045%, which is virtually undetectable.

The expression level of Foxp3, CD25, intracellular CTLA-4, surface CTLA-4, and PD-1 were highest in DP T cells, slightly lower in CD4$^+$ T cells, and lowest in CD8$^+$ and DN T cells (Figures 2A-2–D-2; Figure 3A). When it came to the Foxp3$^+$ and Foxp3$^-$ subpopulations of the four T cell subsets, the expression levels of these molecules were always higher in Foxp3$^+$ cells than Foxp3$^-$ cells with the highest level in Foxp3$^+$ DP cells and slightly lower in Foxp3$^+$CD4$^+$ T cells (Figure 3).

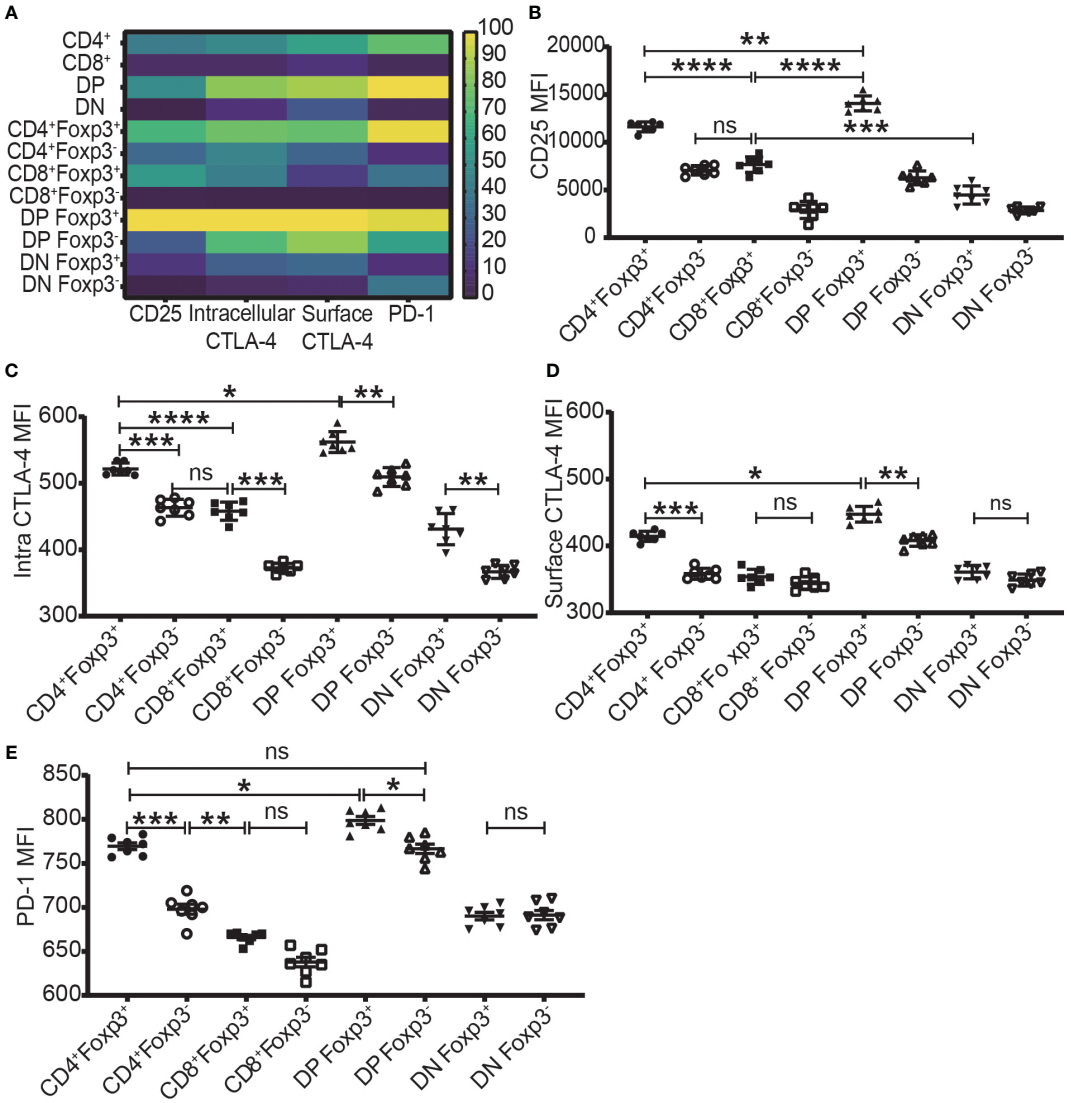

FIGURE 3
The expression levels of CD25, CTLA-4, and PD-1 in the four T cell subsets and their Foxp3$^+$ and Foxp3$^-$ subpopulations. **(A)** shows a heatmap of the expression levels of CD25, intracellular CTLA-4, surface CTLA-4, and PD-1 in the four T cell subsets corresponding to A-2 to E-2 in Figure 2 and in their Foxp3$^+$ and Foxp3$^-$ subpopulations corresponding B to E of this Figure. The values of each parameter were normalized across T cell subsets and their subpopulations, but not across different parameters, meaning that comparison can only be performed on one parameter between T cell subsets (vertically) and their subpopulations but cannot performed on multiple parameters in one subset (horizontally). The normalized score is between 1 to 100. **(B–E)** show the expression level of CD25, intracellular CTLA-4, surface CTLA-4, and PD-1 in Foxp3$^+$ and Foxp3$^-$ subpopulations of the four T cell subsets. The data demonstrated is from lymph nodes of normal C57BL/6 mice and is shown as Mean ± SD (n = 7). Multiple-group comparison was performed by paired One-way ANOVA. The data in spleen shows a similar pattern. For the range of values from $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ were shown as *, **, *** and **** respectively. Ns, not significant.

3.2 The expression of Foxp3 did not completely overlap with the expression of CD25

As shown in Figure 4A–D, the expressions of CD25 and Foxp3 did not completely overlap in any of the four T cell subsets. The overlap of CD25 and Foxp3 expression in CD8$^+$ and DN T cells was poorer than in CD4$^+$ and DP T cells. The highest frequency of Foxp3$^+$ cells in CD25$^+$ cells was in the CD4$^+$ T cell subset and then lower in DP T cells while the percentages of Foxp3$^+$ cells in CD25 positive CD8$^+$ and DN T cells were very low (Figure 4E). These data indicated that about 30%, 50%, 80%, and 97% of CD25$^+$ cells were Foxp3 negative in CD4$^+$, DP, CD8$^+$ and DN T cells respectively. This suggested that Treg purification by CD25 would inevitably cause dramatic contamination of CD25$^+$ Tconv which would be preferably expanded by low-level IL-2 treatment. About 80% of Foxp3$^+$ cells in CD4$^+$ T cells and DP T cells were CD25 positive while these percentages in CD8$^+$ and DN T cells were about 50% (Figures 4F–I). These data showed that there was also a sizeable proportion of Tregs that did not express CD25 and would not benefit from low-level IL-2 treatment.

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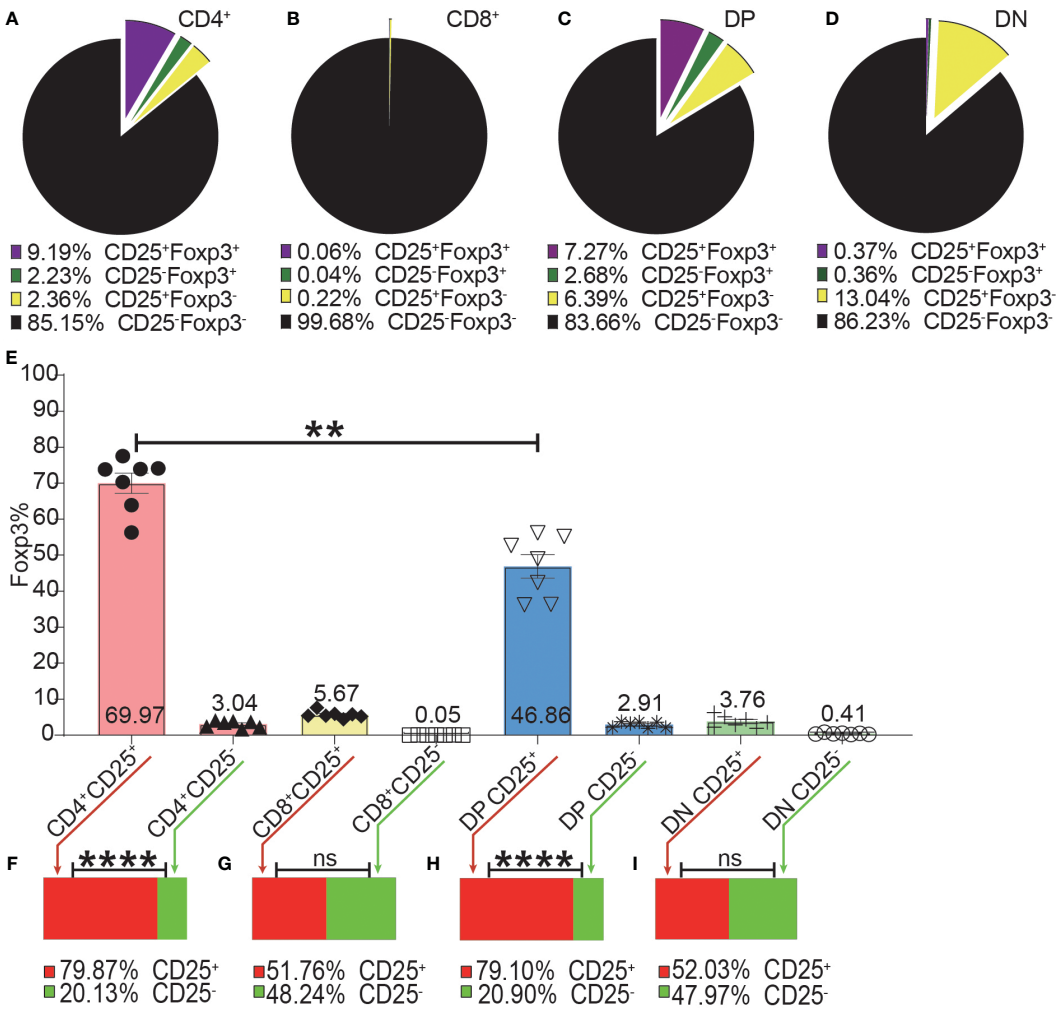

FIGURE 4
The incomplete overlapping between Foxp3 and CD25 expression in the four T cell subsets. The pie charts of **(A–D)** show the proportions of $CD25^+Foxp3^+$, $CD25^-Foxp3^+$, $CD25^+Foxp3^-$, $CD25^-Foxp3^-$ in the four T cell subsets. The data shows the Mean from 7 mice. **(E)** shows the frequencies of $Foxp3^+$ cells in the $CD25^+$ and $CD25^-$ subpopulations of the four T cell subsets, which were generated by placing a CD25 gate on each of the four T cell subsets and then placing a Foxp3 gate on each of the generated $CD25^+$ and $CD25^-$ subpopulations. The bar charts of **(F–I)** show the distribution of $Foxp3^+$ cells of each T cell subset into their $CD25^+$ and $CD25^-$ subpopulations, which was generated by placing a Foxp3 gate on each of the four T cell subsets and then placing a CD25 gate on each of the generated $Foxp3^+$ subpopulations. The data in **(E)** is shown as Mean ± SD and the data in bar charts is shown as Mean, n = 7. The data demonstrated is from lymph nodes of normal C57BL/6 mice. The data in spleen shows similar pattern. For the range of values from $p \leq 0.01$ and $p \leq 0.001$ were shown as ** and *** respectively. Ns, not significant.

3.3 The expression of CTLA-4 or PD-1 did not completely overlap with the expression of Foxp3 and CD25

As Foxp3, CD25, CTLA-4, and PD-1 were mostly expressed in the $CD4^+$ T cell subset, we focused our analysis on $CD4^+$ T cells. Not all intracellular CTLA-4^+, surface CTLA-4^+ or PD-1^+ T cells were CD25 or Foxp3 positive, and a significant proportion of them were CD25 and Foxp3 double negative (Figures 5A-1–C-1, A-2–C-2), which suggested that at least part of CTLA-4 and PD-1 might work independently of both Foxp3 and CD25.

The frequencies of intracellular CTLA-4^+, surface CTLA-4^+ and PD-1^+ were all higher in Tregs than in Tconv (Figures 5D-1–F-1). Our data showed only about 20% of Tregs expressed CTLA-4 intracellularly, and only about 2% of Tregs expressed CTLA-4 on the cell surface (Figures 5D-1, E-1). In other words, although it was shown that CTLA-4 controls Treg function (12), only a small proportion of Tregs expressed CTLA-4 intracellularly and a tiny fraction of Tregs expressed CTLA-4 on the cell surface. In addition, this also suggested that not all Tregs expressing CTLA-4 intracellularly expressed CTLA-4 on the cell surface at the same time. The distribution of intracellular CTLA-4^+ cells and surface CTLA-4^+ cells was greater in the Treg than in the Tconv compartment (Figure 5D-2, E-2) while more PD-1^+ T cells were distributed in the Tconv than in the Treg compartment (Figure 5F-2), which showed that CTLA-4 and PD-1 are constitutively expressed by a small proportion of both Tregs and Tconv in the homeostatic state.

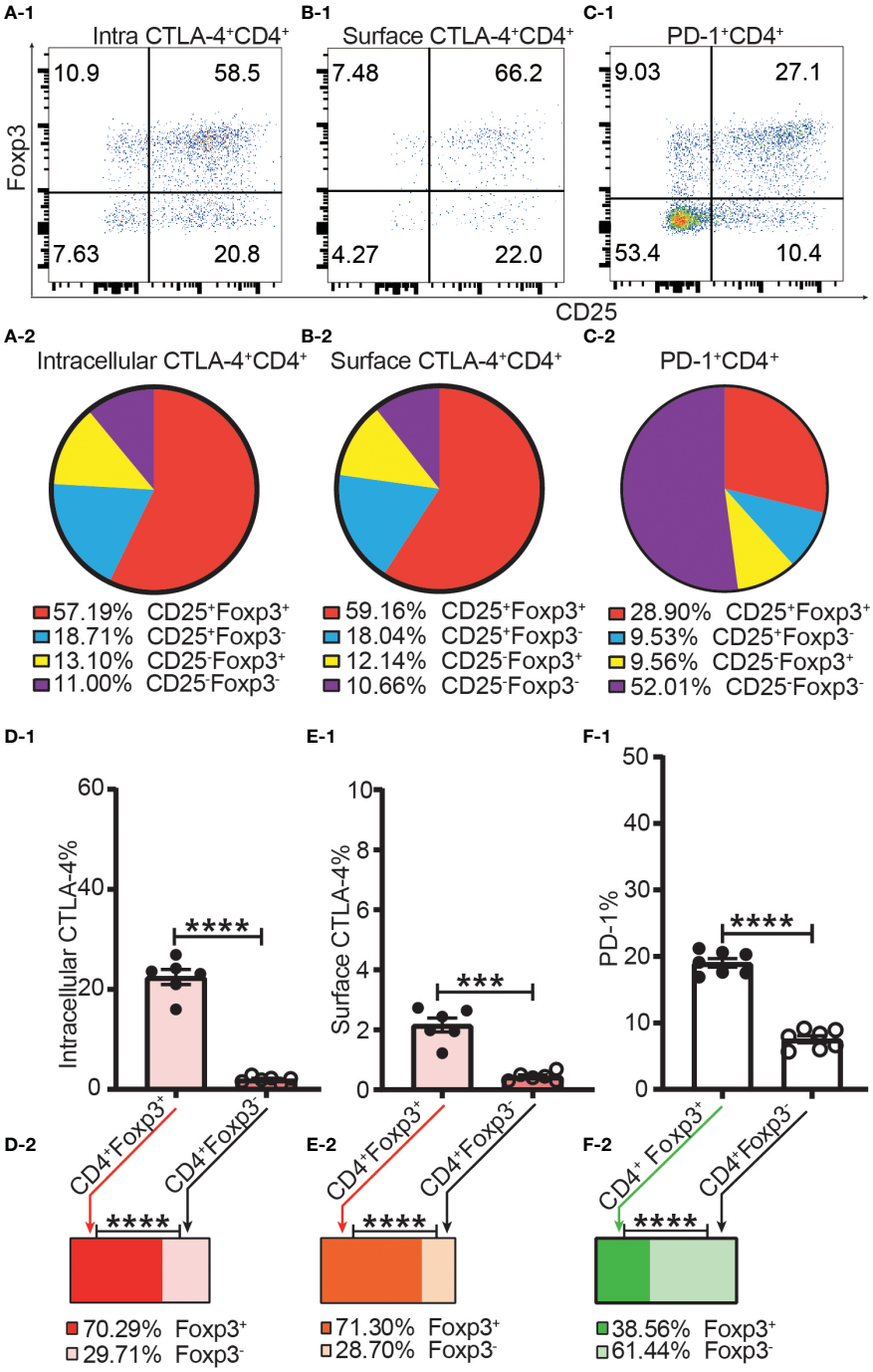

FIGURE 5
The incomplete overlapping between the expression of Foxp3 and the expression of CTLA-4 and PD-1 in $CD4^+$ T cells. Dot plots **(A-1)**, **(B-1)**, and **(C-1)** show the distribution of intracellular CTLA-4^+CD4^+ T cells, surface CTLA-4^+CD4^+ T cells and PD-1^+CD4^+ T cells respectively into $CD25^+Foxp3^+$, $CD25^+Foxp3^-$, $CD25^-Foxp3^+$, and $CD25^-Foxp3^-$ compartments of $CD4^+$ T cells. Pie charts of **(A-2)**, **(B-2)**, and **(C-2)** shows the Mean of **(A-1)**, **(B-1)**, and **(C-1)** respectively (n = 7). **(D-1)**, **(E-1)**, and **(F-1)** show the percentage of intracellular CTLA-4^+, surface CTLA-4^+, and PD-1^+ cells in Tregs and Tconv respectively. The bar charts of (D-2), **(E-2)**, and **(F-2)** show the distribution of intracellular CTLA-4^+CD4^+ cells, surface CTLA-4^+CD4^+ cells, and PD-1^+CD4^+ cells respectively into Treg and Tconv compartments. The data in A-2 to C-2 and D-2 to F-2 are shown as Mean and the data in D-1 to F-1 are shown as Mean $\pm$ SD, n =7. The data demonstrated is from lymph nodes of normal C57BL/6 mice and the data in spleen has the similar pattern. For the range of values from $p \leq 0.001$ and $p \leq 0.0001$ were shown as *** and **** respectively.

3.4 Dynamics of expression of Foxp3, CD25, CTLA-4 and PD-1 after skin transplantation in mice

As will be discussed in 3.5, T cells were not activated at day 3 but had been activated at day 7 post-transplantation.

To examine whether the percentage of Foxp3$^+$ cells increased after allo-activation, we employed both mRFP-Foxp3 reporter mice and normal C57BL/6 mice as recipients of B6D2F1 skin transplants. The expression levels of mRFP and Foxp3 both had been significantly upregulated at day 7 after skin transplantation (Figures 6A-1, B-1). However, the percentage of mRFP$^+$ cells in CD4$^+$ T cells at day 7 (only day 7 timepoint was observed for mRFP reporter T cells) and the percentage of Foxp3$^+$ cells in CD4$^+$ T cells at day 7 and day 14 did not significantly change (Figures 6A-2, B-2). As Foxp3 positive CD8$^+$ Tregs were also reported (16, 17), we then checked Foxp3 expression in CD8$^+$ T cells after allo-activation and found that the low percentage of Foxp3$^+$ cells in CD8$^+$ T cells did not significantly increase (Figure 6C).

It is generally accepted that CD25 is upregulated in Tconv after activation and that it is not reliable to be used as a sorting marker for Tregs, especially after CD4$^+$CD25$^+$ T cell expansion. Our data showed that although the CD25 expression level increased, the percentage of CD25$^+$CD4$^+$ T cells did not significantly increase at day 7 and day 14 after transplantation (data not shown). When explored further, same as in CD4$^+$ T cells, although the CD25 expression level increased, the percentage of CD25$^+$ cells in Tregs and Tconv also did not significantly increase at day 7 and day 14 (Figures 6D-1, D-2). These data suggested that CD25 expression could not be induced from CD25 negative cells, at least in the model of mouse skin transplantation.

The expression level and percentage positive of CTLA-4 (both intracellular and surface) and PD-1 had been upregulated at day 7 (Figures 6E-1, E-2, F-1, F-2). Like resting T cells, the intracellular CTLA-4 level was higher than surface CTLA-4 in both Tregs and Tconv and increased after T cell activation (Figure 6E-1). The expression of PD-1 and intracellular CTLA-4 in Tregs and Tconv was significantly upregulated at day 7 and day 14 timepoints (Figures 6E-1, F-1). Although slightly increased, the surface CTLA-4 expression level and percentage positive in both Tregs and Tconv were always maintained at a low level (Figures 6E-1, E-2). Compared with the day 7 timepoint, the expression level of surface CTLA-4 slightly dropped at day 14 (Figure 6E-1). The percentages of intracellular CTLA-4$^+$ and PD-1$^+$ cells both in Tregs and Tconv increased from day 7 (Figures 6E-2, F-2), which indicated that CTLA-4 and PD-1 expression could be induced from CTLA-4$^-$ cells and PD-1$^-$ cells respectively after T cell activation.

3.5 Foxp3$^+$ T cells maintained a higher ratio of proliferating cells than Foxp3$^-$ T cells in the homeostatic state and after allo-activation

Proliferation was assessed by Ki-67 and Ki-67$^+$ T cells were defined as proliferating and activated T cells. Both Foxp3$^+$ cells and Foxp3$^-$ cells of the four T cell subsets maintained a low level of Ki-67 expression and low ratio of Ki-67$^+$ cells in the homeostatic state (Figures 7A, B). The Ki-67 expression level and Ki-67$^+$ percentage were both higher in Foxp3$^+$ cells than in Foxp3$^-$ cells (Figures 7A, B). The average ratios of proliferation in CD4$^+$Foxp3$^+$ Tregs and CD4$^+$Foxp3$^-$ Tconv were 15.25% and 1.89% respectively (Figure 7B).

The Ki-67 expression level and Ki-67$^+$ percentage in Tregs and Tconv did not significantly increase at day 3 but had increased at day 7 (Figures 7C-1–F-1, C-2–F-2, G, H), which indicated that T cells began to proliferate at a timepoint between day 3 and day 7 in the murine skin transplant model. The endogenously expanded Tregs maintained a slightly higher proliferating percentage than in Tconv after allo-activation at day 7 and day 14 (Figure 7G, H).

About half of the proliferating cells in CD4$^+$ T cells were distributed in the Foxp3$^+$ compartment before allo-activation at day 0 and day 3 (Figures 7I-1, I-2, J-1, J-2), which suggested that the ratio of functioning self-reactive Tregs and Tconv might be about the same number in the normal homeostatic state. This is in contrast to the assumption that the 5% to 10% Tregs counterbalance the majority of Tconv (18). We then calculated the ratio of proliferating allo-reactive Treg/Tconv in the Ki-67$^+$CD4$^+$ T cells at day 7 and day 14. The average ratios at day 7 and day 14 were 1:5.92 and 1: 6.98, respectively (Figures 7K-1, K-2, L-1, L-2). This indicated that allo-reactive Tregs were dominated by allo-reactive Tconv in transplant rejection and about 5 to 6-fold more of activated allo-reactive Tregs might be required to achieve transplant tolerance.

3.6 Tregs and Tconv exhibit different proliferation characteristics *in vivo* and *in vitro*

The *in vivo* and *in vitro* proliferation of Tregs and Tconv was assessed by flow cytometric analysis of CFSE at day 3 after adoptive transfer and cell culture. Different application settings were used for the flow cytometric analysis of *in vitro* and *in vivo* proliferation. The gating strategies for Tregs and Tconv *in vivo* and *in vitro* are shown in Supplementary Figures 2A–D.

In the *in vivo* expansion model, donor cells consisting of pooled B6Ly5.1 CD4$^+$ T cells from lymph nodes and spleen were injected into B6D2F1 mice on a one-donor-to-two-recipient basis. Both donor Tconv and Tregs proliferated after encountering allo-antigens in lymph nodes and spleen of the cell recipient mice (Figures 8A-1, A-2, B-1, B-2). Among the expanded cells, the leftmost peak was defined as highly proliferating cells. There were as many as 6 peaks present, which meant that part of the donor cells had divided 6 times and the cell number expanded by $2^6 = 64$ times. The vigorous *in vivo* T cell expansion indicated that the cells were not functionally compromised by the *in vitro* operations to prepare cells for injection including red blood cell lysis and magnetic selection. The percentage of donor CD4$^+$ T cells accounted for 10.40% to 19.29% (14.35 ± 3.71) of total CD4$^+$ T cells from recipient lymph nodes. This percentage range in the spleen was from 24.8% to 39.55% (31.14 ± 5.44). The proportion of donor cells in spleen was significantly higher than in lymph nodes. This might be, at least partially, due to the fact that in preparation of donor cells for

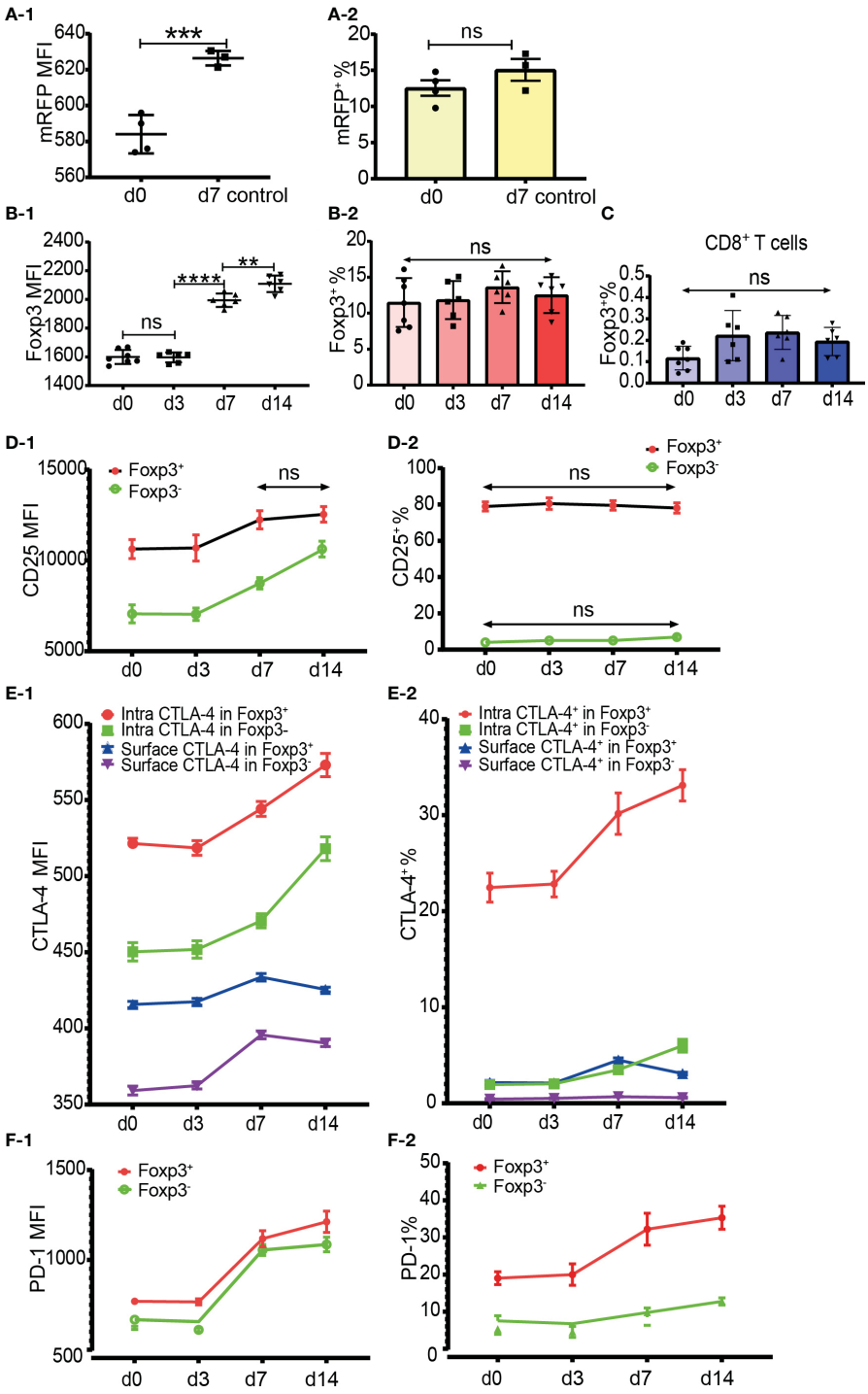

FIGURE 6
Dynamics of Foxp3, CD25, CTLA-4 and PD-1 expression after skin transplantation. **(A-1, A-2)** show mRFP expression level and percentage positive of mRFP$^+$ T cells respectively in CD4$^+$ T cells at day 0 (non-transplanted) and day 7 from draining lymph nodes. The data was displayed as Mean ± SD, n =4 at day 0 (non-treated) and n = 3 at day 7. **(B-1, B-2)** show Foxp3 expression level and percentage positive of Foxp3$^+$ T cells respectively in CD4$^+$ T cells at day 0, 3, 7 and 14. **(C)** shows the percentage positive of CD8$^+$Foxp3$^+$ T cells at day 0, 3, 7, and 14. **(D-1, D-2)** show CD25 expression level and percentage of CD25$^+$ cells in Tregs and Tconv at day 0, 3, 7, and 14. **(E-1, E-2)** show CTLA-4 expression level and percentage positive of CTLA-4$^+$ cells in Tregs and Tconv at day 0, 3, 7, and 14. **(F-1, F-2)** show PD-1 expression level and percentage positive of PD-1$^+$ cells in Tregs and Tconv at day 0, 3, 7, and 14. The data is displayed as Mean ± SD, n =7 at day 0, n = 6 at day 3, 7, and 14. Two-group comparison was performed using unpaired t test and multiple-group comparison was performed using unpaired one-way ANOVA. For the range of values from $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ were shown as **, *** and **** respectively. Ns, not significant.

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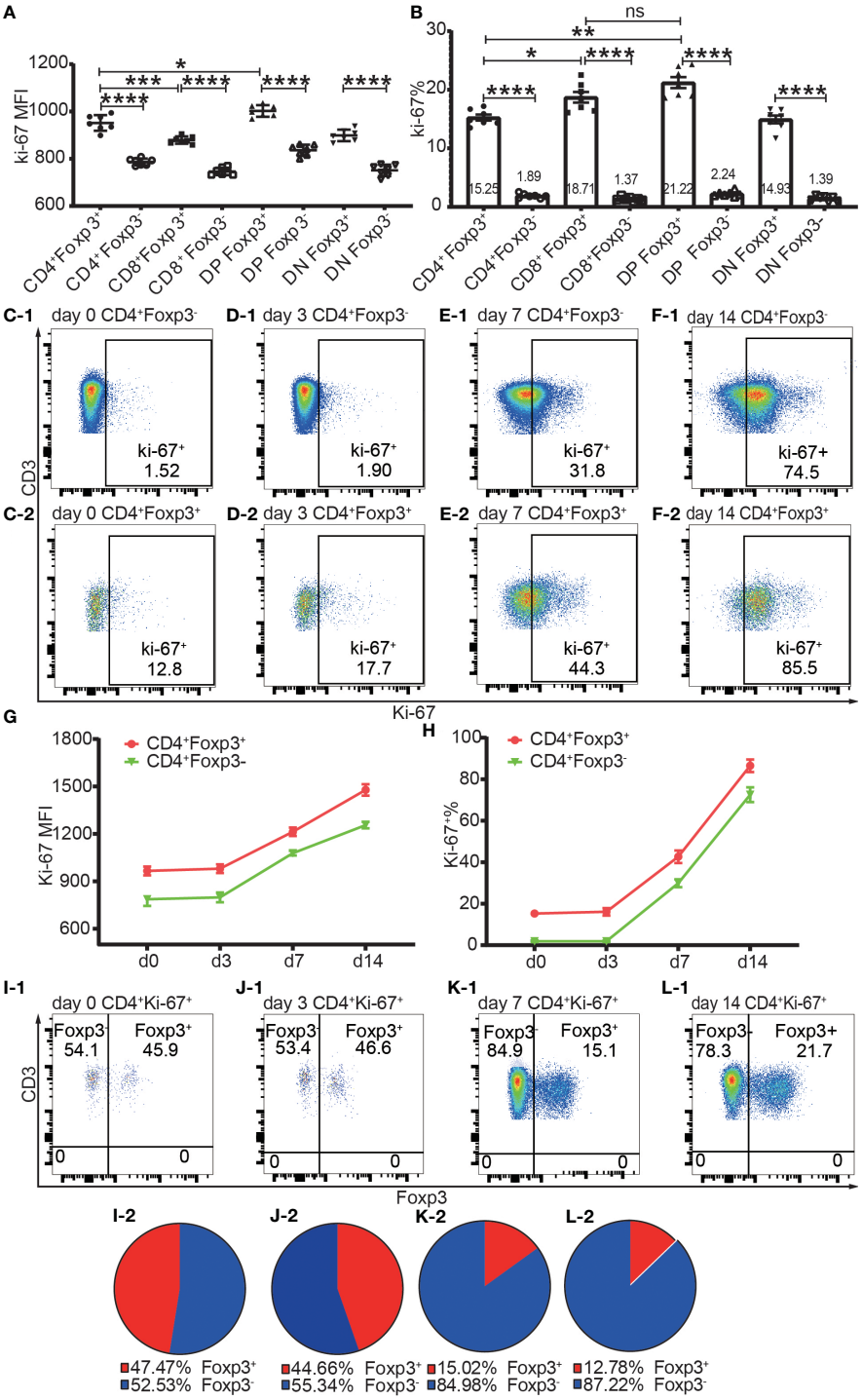

FIGURE 7
The expression of Ki-67 in Foxp3$^+$ and Foxp3$^-$ T subpopulations in the homeostatic state and after allo-activation. The proliferation was assessed by Ki-67 expression and the data is from draining lymph nodes. **(A. B)** show the expression level of Ki-67 and percentage of Ki-67$^+$ cells respectively in Foxp3$^+$ and Foxp3$^-$ subpopulations of the four T cell subsets at day 0 (non-transplanted). The data is shown as Mean ± SD, n = 7. **(C-1, D-1, E-1, F-1)** show representative dot plots of percentages of Ki-67$^+$ cells in Tconv at day 0, 3, 7, and 14 respectively. **(C-2, D-2, E-2, F-2)** show representative dot plots of percentages of Ki-67$^+$ cells in Tregs at day 0, 3, 7, and 14 respectively. **(G, H)** show the expression levels of Ki-67 and percentages of Ki-67$^+$ cells in Tconv and Tregs at day 0, 3, 7, and 14. The data is shown as Mean ± SD, n =7 at day 0, n = 6 at day 3, 7, and 14. The dot plots of **(I-1, J-1, K-1, L-1)** show the distribution of Ki-67$^+$CD4$^+$ T cells into Tconv and Treg compartments at day 0, 3, 7, and 14. The pie charts of **(I-2, J-2, K-2, L-2)** show the Mean of **(I-1, J-1, K-1, L-1)** respectively, n =7 at day 0, n = 6 at day 3, 7, and 14. For the range of values from $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ were shown as *, **, *** and **** respectively.

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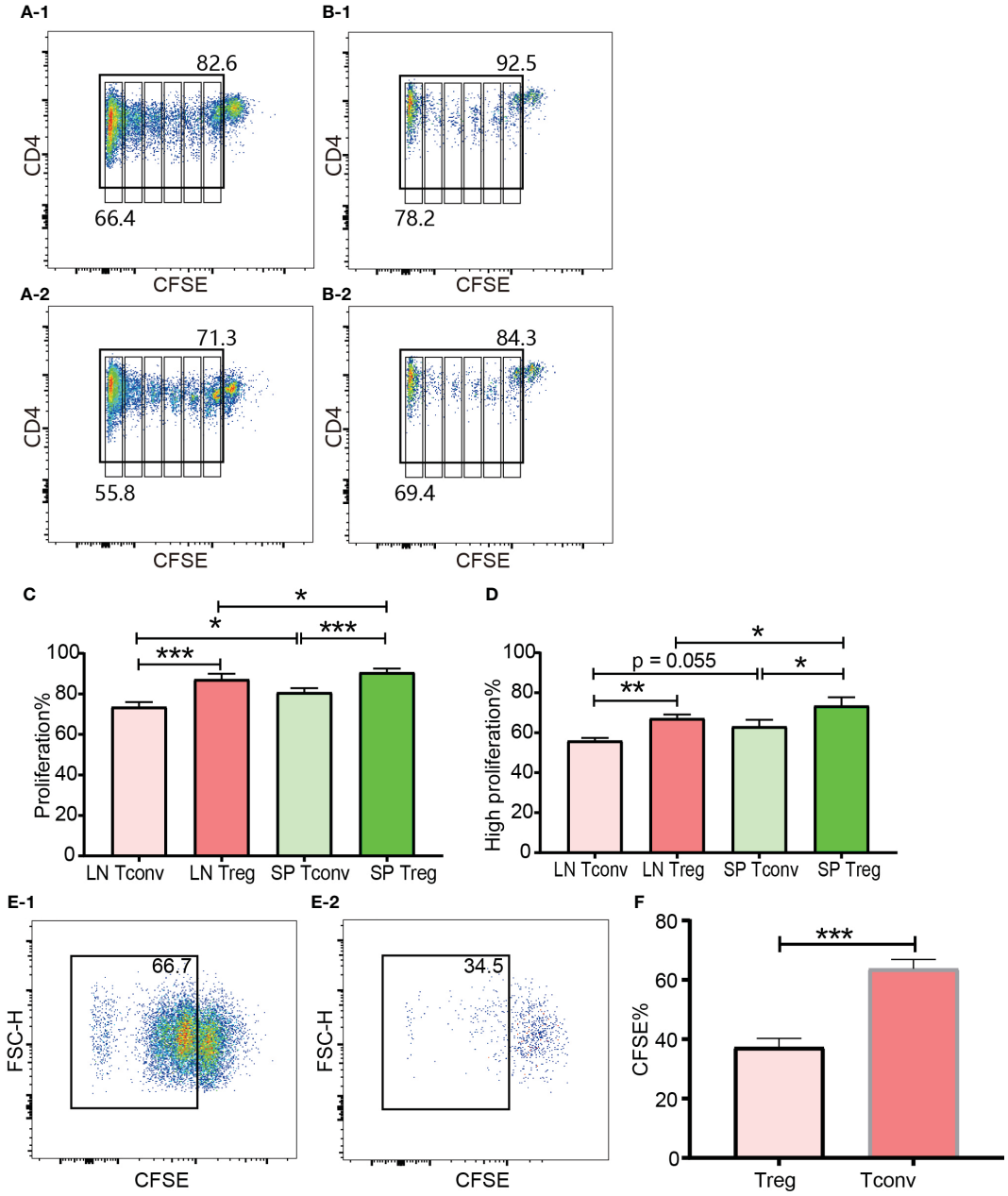

FIGURE 8
In vivo and *in vitro* expansion of Tregs and Tconv at day 3. The proliferation was assessed by CFSE. **(A-1, B-1)** show the proliferation of donor Tconv and Tregs respectively in recipient spleen. **(A-2), (B-2)** show the proliferation of donor Tconv and Tregs respectively in recipient lymph nodes. The rightmost column was non-proliferating cells, and the leftmost column was defined as highly proliferating cells. Six cell division peaks can be identified in both of Tconv and Tregs. **(C)** shows the percentages of proliferating Tregs and Tconv. **(D)** shows the percentages of highly proliferating Tregs and Tconv. The data was shown as Mean ± SD, n = 3. The comparison between two groups was performed by paired t test. **(E-1, E-2)** show the proliferation of Tconv and Tregs *in vitro* at day 3. Two ambiguous division peaks can be identified in both of Tconv and Tregs. All these dividing cells were defined as proliferating cells. **(F)** is comparison of the percentage of proliferating Tregs and Tconv *in vitro*. The data in **(F)** was displayed as Mean ± SD, n = 3. The comparison between two groups was performed by paired t test. For the range of values from $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ were shown as *, ** and *** respectively.

injection more cells were generated from the spleen than from the lymph nodes, and these cells were inclined to recirculate to the lymphoid organs from which they were derived. Another possibility was that the cells were adoptively transferred via the blood stream and would reach the spleen earlier than the lymph nodes. Whether in the lymph nodes or spleen, donor Tregs maintained a slightly higher percentage of proliferation than donor Tconv (Figure 8C, D). The higher ratio of proliferating cells in Tregs than in Tconv after allo-activation was consistent with the data assessed using Ki-67 in skin transplant models discussed in subheading 3.5.

The cells for *in vitro* cell culture and *in vivo* adoptive transfer underwent the same preparations (e.g., physical disruption of the lymph nodes and spleen for cell isolation, blood cell lysis, magnetic $CD4^+$ isolation, and CFSE labelling). Although published protocols

were closely followed both Tregs and Tconv proliferated very poorly in the presence of irradiated B6D2F1 allo-splenocytes (Figures 8E-1, E-2). Except the left most column (probably background) there were two poorly defined dividing peaks present, which meant that a small proportion of T cells had expanded as many as 4 times. This was in contrast to the *in vivo* proliferation of Tregs and Tconv and indicated that the *in vitro* behaviour of Tregs and Tconv was not representative of their behaviour *in vivo*, which was probably because it was difficult to perfectly mimic the *in vivo* conditions *in vitro*. In regard to the relative proliferation of Tregs compared with Tconv, the proliferating percentage in Tregs was significantly less *in vitro* (Figure 8F).

3.7 Differential gene expression revealed that naïve Tregs had similar molecular characteristics to activated Tconv

Tregs ($CD4^+mRFP^+Foxp3^+$) and Tconv ($CD4^+mRFP^-Foxp3^-$) sorted from non-transplanted mRFP mice were termed "naïve Tregs" (n = 4) and "naïve Tconv" (n = 4) respectively. As the flow cytometry data confirmed that T cells had been activated at day 7 after transplantation, Tregs and Tconv sorted from allogeneic skin recipient mRFP mice at day 7 were termed "activated Tregs" (n = 3) and "activated Tconv" (n = 3) respectively. The heatmap of all the 14 samples is shown in Figure 9A.

The differentially expressed genes between naïve Tregs and naïve Tconv screened by adjusted p < 0.05 are shown in Figure 9B and Supplementary Data 1. Consistent with the flow cytometry data the expression of CD25, CTLA-4, and PD-1 encoding genes *Il2ra*, *Ctla4*, and *Pdcd1* were all higher in Tregs than in Tconv. The top 5 gene sets for the differentially expressed genes were IL-10, T cell checkpoint, T cell exhaustion, IL-2, and CTLA-4. The expressions of co-stimulatory and co-inhibitory molecule-encoding genes was all higher in naïve Tregs than in naïve Tconv. The co-stimulatory molecule-encoding genes also included those responsible for activation-induced molecules such as ICOS and LAG3. Not only genes for co-inhibitory molecules (e.g., *Ctla4* and *Pdcd1*) and immunosuppressive cytokines (e.g., *Il10*), but also genes for their ligands or receptors (e.g., *Cd86, Cd274, Pdcd1lg2* and *Il10rb*) were higher in Tregs than in Tconv. The gene

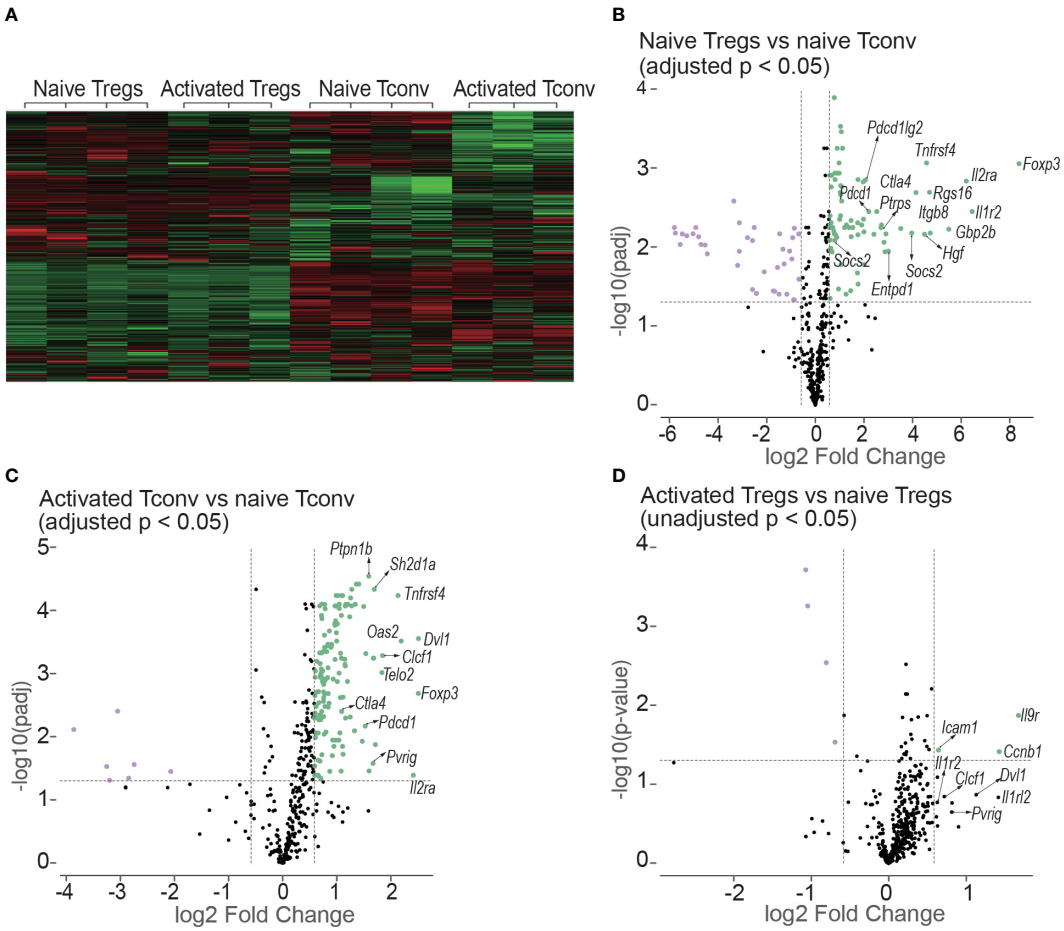

FIGURE 9
Heatmap and volcano plots in differential gene expression analysis. **(A)** shows the heatmap of normalized values of all the 14 samples: naive Tregs (n =4); naive Tconv (n = 4); activated Tregs (n = 3); activated Tconv (n = 3). The volcano plots above the dotted horizontal line show the differentially expressed genes between naïve Tregs and naïve Tconv **(B)**; between activated Tconv and naïve Tconv **(C)**; between activated Tregs and naïve Tregs **(D)**. The differentially expressed genes were screened using adjusted p < 0.05 in **(B, C)** but using unadjusted p < 0.05 as no genes were screened using adjusted p < 0.05. The vertical lines represent the 1.5-fold change.

sets were generally classified into two groups. The first group was defined as T cell negative regulation related genes which contained co-inhibitory, anergy, senescence & quiescence, apoptosis, and immunosuppressive cytokine- associated genes. Except for immunosuppressive cytokines, all these mechanisms are proposed to be involved in negative regulation of conventional T cells and maintaining peripheral tolerance (reviewed in (19)). For the immunosuppressive cytokines such as IL-10 and TGF-β, they are typically expressed in Tregs and important for Treg-mediated immunosuppression. The second group was defined as T cell activation related gene sets which contains T cell activation (TCR, co-stimulatory and inflammatory cytokine), intracellular signal transduction molecules (PI3K-AKT, JAK/STAT, NF-κB, MAKP, Notch and mTOR), and cell cycle related genes. In addition, the genes for suppressor of cytokines such as *Cish, Socs2*, and *Socs3* were expressed higher in Tregs than in Tconv.

The differentially expressed genes between activated Tconv and naïve Tconv (adjusted $p < 0.05$) were shown in Figure 9C and Supplementary Data 2. Some typical T cell activation marker encoding genes such as *Cd69* and *Cd101* were upregulated and the gene for CD62L (*Sell*) was downregulated in Tconv at day 7 after skin transplantation, which indicated that consistent with the flow cytometry data assessed by Ki-67, T cells had been allo-activated at this timepoint. From the gene sets of antigen presentation, TCR and T cell checkpoint, the genes encoding MHC molecules (e.g., *H2-d1, H2-k1, H2-m3, H2-t23*), TCR and its activation associated molecules (e.g., Cd3d, *Trat1, Trbc1/2, Trdv2-1/2-2, Zap70*), activation-induced molecules (e.g., *Icos, Tnfrsf14, Tnfrsf18, Tnfrsf4*), and co-inhibitory molecules (e.g., *Ctla4* and *Pdcd1*) as well as ligands of PD-1 (*Cd274* and *Pdcd1lg2*) were upregulated. In addition, the gene expression of major functional molecules of various intracellular signal transduction pathways including AP-1, mTOR, Notch, MARP, JAK/STAT, NF-κB, and PI3K-AKT increased.

For the comparison between activated Tregs and naïve Tregs, no differentially expressed genes were obtained by adjusted $p < 0.05$. Instead, the differentially expressed genes were screened using unadjusted p value < 0.05 as shown in Figure 9D and Supplementary Data 3. However. the genes encoding molecules for T cell activation and T cell checkpoint and intracellular signal transduction present in activated Tconv were not present in Tregs after allo-activation.

4 Discussion

4.1 Foxp3 and CD25 expression in $CD8^+$ T cells

The percentages of both $Foxp3^+$ and $CD25^+$ cells were very low and even undetectable in $CD8^+$ T cells. These percentages did not significantly increase after allo-activation. In addition, overlap of Foxp3 and CD25 expression in $CD8^+$ T cells was very poor. Although $CD8^+CD25^+$ (20, 21), $CD8^+Foxp3^+$ (22), and $CD8^+CD25^+Foxp3^+$ (23, 24) T cells were shown to be immunosuppressive and have been proposed as $CD8^+$ Tregs, these populations are numerically small and are likely to be more challenging than $CD4^+$ Tregs to translate to clinical therapy.

4.2 Peripheral DP T cells were characteristic of $CD4^+$ T cells but with higher basal Ki-67 expression

DP T cells have been extensively studied during their development in the thymus but are less well-characterised in the periphery. For thymic DP cells, Foxp3 (2, 4) and CD25 (25) have not been detected. Our data showed that both Foxp3 and CD25 expression were higher in peripheral DP T cells than in other T cell subsets, indicating that the peripheral DP T cells are different from those immature thymic DP T cells. Not only Foxp3 and CD25, but also the expressions of intracellular CTLA-4, surface CTLA-4, and PD-1 as well as the proliferation marker Ki-67 were highest by peripheral DP T cells among the four T cell subsets (Figure 2). Hence, we speculated that peripheral DP T cells might be an activated cell population. As DP T cells were reported to dramatically increase in certain diseases (reviewed in (26)) and *in vitro* induced $CD4^+CD8^+Foxp3^+$ from $CD4^+Foxp3^+$ T cells were more immunosuppressive (27), this unique T cell subset deserves more attention either for diagnosis value or for therapeutic purposes.

4.3 The incomplete overlapping between the expression of Foxp3 and CD25

Although it is widely accepted that Foxp3 has been the best marker for Tregs, the definition of regulatory T cells has been inconsistent. For example, the term "Tregs" is interchangeably used for $CD4^+Foxp3^+$ T cells, $CD4^+CD25^+Foxp3^+$ T cells, and $CD4^+CD25^+$ T cells. This presupposes the complete co-expression of CD25 and Foxp3 in $CD4^+$ T cells. The incomplete overlap of the expression of CD25 and Foxp3 from our data indicated that it might present problems for both adoptive Treg therapy and low-level IL-2 treatment for immune tolerance induction. For adoptive Treg therapy, "Tregs" normally refers to $CD4^+CD25^+$ T cells, especially in mice. Our data showed that the purity of $CD4^+Foxp3^+$ T cells in $CD4^+CD25^+$ T cells was about 70%, this percentage in published data from GFP reporter mice was even less, about 50% (4). In addition, our data also showed that Tconv preferably expanded during *in vitro* expansion. Hence, it is possible that the big proportion of contaminating $CD4^+Foxp3^-CD25^+$ effectors might exacerbate the conditions instead of achieving therapeutic purposes. For low-level IL-2 treatment, it is generally believed that CD25, as a unique sub-unit of the IL-2 receptor making Tregs more sensitive to IL-2, is specially expressed on Tregs in the homeostatic state. Using Foxp3 as the definitive Treg marker, the large proportion of $CD25^+Foxp3^-$ Tconv will also be activated and expanded along with Tregs in the presence of low-level of IL-2. This might reduce the efficacy of the inducted Tregs and might even lead to a poorer outcome, especially in strongly inflammatory conditions. Hence, although Treg-based therapies have been shown to be helpful for

transplant tolerance induction in animal models, our data suggested that stand-alone adoptive "Treg" therapy or standalone low-level IL-2 therapy might be difficult to move to the clinic.

4.4 The expression of CTLA-4 and PD-1 by Tregs and Tconv

Our flow cytometry data showed that the expression of Foxp3 did not completely overlap with the expression of CTLA-4 and PD-1. In other words, although at a higher level in Tregs, both CTLA-4 and PD-1 were constitutively expressed in a small proportion of Tregs and Tconv in the homeostatic state. This suggested that CTLA-4 and PD-1 might work both in Tregs and Tconv to inhibit the activation of the effector arm, although the role of CTLA-4 and PD-1 in Treg-mediated immune-regulation needs to be better defined. CTLA-4 was shown to be crucial for Treg function (12) and has been proposed as a marker to identify regulatory T cells (reviewed in (14)), In contrast, our data showed that only a small proportion of Tregs expressed CTLA-4 even intracellularly and not every Treg expressing CTLA-4 intracellularly also expressed it on the cell surface. Although slightly increased after activation, the expression level and the percentage positive of surface CTLA-4 was maintained at a very low level on Tregs, which suggested that the vast majority of Tregs might not rely on CTLA-4 to mediate immunosuppression.

4.5 The higher levels of CTLA-4 and PD-1 in Tregs did not negatively correlate with proliferation compared to Tconv

CTLA-4 and PD-1 serve as co-inhibitory receptors which inhibit T cell activation or increase the activation threshold of T cells. However, the role of CTLA-4 and PD-1 in Treg activation is not fully understood. Although the expression levels of CTLA-4 and PD-1 were higher in/on Tregs than in/on Tconv, there were two unexpected findings: Tregs maintained a higher level of Ki-67 expression in the immune homeostatic state and had greater *in vivo* expansion than Tconv after allo-stimulation in both the adoptive transfer experiment and the skin transplant experiment. This suggested that CTLA-4 and PD-1 did not negatively correlate with Treg activation. Hence, we speculated that either CTLA-4 and PD-1 function differently in Tregs than in Tconv or naïve Tregs were already in an activated state.

4.6 Treg specificities

Our data showed that both Tregs and Tconv maintained a basal proliferation as assessed by Ki-67 expression in the homeostatic state. It is possible that this basal proliferation is triggered by self-antigens and identifies T cells that are self-reactive. Although there is some conflicting evidence, the question of whether Tregs are self-reactive or non-self-reactive is still unsettled (reviewed in (14)). Our data showed that one quarter of Tregs and two percent of Tconv were proliferating as measured by their expression of Ki-67. This might be driven by their self-reactivity. In other words, our data supported the argument that most Tregs are non-self-reactive (reviewed in (14)). Contrary to the belief that Tregs are all self-reactive, the non-self-reactivity makes it theoretically feasible for transplant tolerance induction.

4.7 The "activated" molecular characteristics of Tregs

CD25 and Ki-67 are commonly used as T cell activation markers and the co-inhibitory molecules, CTLA-4 and PD-1, are upregulated after T cell activation. In this sense, the high expression of these molecules makes Tregs appear like an "activated" Tconv. Our data from differential gene expression further supported this hypothesis. For example, including genes for CD25, CTLA-4, and PD-1 genes for T cell activation markers, inducible co-inhibitory molecules, and key molecules in a number of intracellular activation pathways all were expressed at a higher level in naïve Tregs than naïve Tconv. This pattern of differentially expressed genes between naïve Tregs and naïve Tconv was similar to that between activated Tconv and naïve Tconv. Furthermore, when compared with naïve Tconv, the genes for the suppressor of cytokines which suppress the expression of inflammatory-cytokine-encoding genes were highly expressed in Tregs. In addition to genes encoding CTLA-4, PD-1, and IL-10, the genes encoding their ligands or receptors (e.g., CD86, PD-L1, PD-L2, and IL10R) were also expressed higher in Tregs than in Tconv, which raises the possibility that Tregs might also be target cells of their own mechanisms of action if they were "activated Tconv". In other words, unless Treg-specific marker(s) are identified from normal animals and humans and Tregs are sufficiently characterised, we cannot preclude another possibility that T cell-mediated immunosuppression is the outcome of self-regulation (reviewed in (14)) in which Tregs might be an over-activated subpopulation which have initiated their negative regulation system such as upregulation of CTLA-4 and PD-1 and release of immunosuppressive cytokines.

In conclusion, our data provides a novel insight into the negative regulation by Tregs and co-inhibitory molecules, the *in vivo* and *in vitro* proliferation characteristics of Tregs compared with Tconv and the molecular characteristics of gene expression in Tregs. However, Treg-specific marker(s) for purification of viable Tregs are badly needed for characterisation and therapeutic studies of Tregs.

Data availability statement

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

Ethics statement

The animal study was approved by The University of Newcastle Animal Care and Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZL: Conceptualization, Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft, Formal Analysis. KB: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. NN: Investigation, Methodology, Writing – review & editing. MH: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. DC: Data curation, Methodology, Supervision, Writing – review & editing. GB: Conceptualization, Formal Analysis, Supervision, Writing – review & editing. PT: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The research was supported by the Hunter Transplant Research Foundation (HTRF) and HDR student support allocation from University of Newcastle, Australia. The NanoString work was supported by 2022 Strategic Research Pilot Grants from the College of Health, Medicine and Wellbeing, The University of Newcastle, Australia.

Acknowledgments

We thank University of Newcastle and China Scholarship Council for their sponsorship for ZL's studies. We thank Associate Professor Alexandra Sharland from the University of Sydney for her generous provision of mRFP mice and preliminary data discussion of the project. We thank Senior Technical Officer Miss Nicole Cole from Analytical Biomolecular Research Facility, University of Newcastle, Australia, for her assistance in flow cytometry.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1292158/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 15 March 2024
DOI 10.3389/fimmu.2024.1331609

OPEN ACCESS

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RECEIVED 01 November 2023
ACCEPTED 05 March 2024
PUBLISHED 15 March 2024

CITATION
Xia Y, Gao D, Wang X, Liu B, Shan X, Sun Y and Ma D (2024) Role of Treg cell subsets in cardiovascular disease pathogenesis and potential therapeutic targets.
Front. Immunol. 15:1331609.
doi: 10.3389/fimmu.2024.1331609

Role of Treg cell subsets in cardiovascular disease pathogenesis and potential therapeutic targets

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In the genesis and progression of cardiovascular diseases involving both innate and adaptive immune responses, inflammation plays a pivotal and dual role. Studies in experimental animals indicate that certain immune responses are protective, while others exacerbate the disease. T-helper (Th) 1 cell immune responses are recognized as key drivers of inflammatory progression in cardiovascular diseases. Consequently, the CD4+CD25+FOXP3+ regulatory T cells (Tregs) are gaining increasing attention for their roles in inflammation and immune regulation. Given the critical role of Tregs in maintaining immune-inflammatory balance and homeostasis, abnormalities in their generation or function might lead to aberrant immune responses, thereby initiating pathological changes. Numerous preclinical studies and clinical trials have unveiled the central role of Tregs in cardiovascular diseases, such as atherosclerosis. Here, we review the roles and mechanisms of Treg subsets in cardiovascular conditions like atherosclerosis, hypertension, myocardial infarction and remodeling, myocarditis, dilated cardiomyopathy, and heart failure. While the precise molecular mechanisms of Tregs in cardiac protection remain elusive, therapeutic strategies targeting Tregs present a promising new direction for the prevention and treatment of cardiovascular diseases.

KEYWORDS

cardiovascular disease, Treg cell, immunotherapy, immune microenvironment, atherosclerosis, hypertension

1 Introduction

Cardiovascular diseases (CVD) stand as the leading global cause of mortality (1, 2). Despite receiving state-of-the-art preventative medical interventions, patients with CVD still face a significant risk of recurrent events. Much of this residual risk is attributed to immune-inflammatory responses (2). T-cell mediated inflammatory reactions have been identified as central in the pathogenesis of CVD (3, 4). Consequently, targeting infiltrating T-cell subsets might offer innovative and promising therapeutic strategies for CVD (5).

A specific subset of infiltrating T cells, the Treg cells, constitute approximately 5-10% of all peripheral CD4+ T cells and play pivotal roles in maintaining homeostasis, immunological balance, and tolerance (6, 7). Aberrant accumulation or functional anomalies of Treg cells are closely associated with autoimmune diseases, chronic inflammation, infection progression, and tumor development. CD4+CD25+Foxp3+ regulatory T (Treg) cells evolve from immature T cells activated by antigens and cytokines (8, 9). While primarily maturing in the thymus, they can also transdifferentiate from peripheral naive CD4+ T cells (10). The forkhead/winged-helix transcription factor (FOXP3) serves as a hallmark for Treg cells, and the regulatory T cells expressing the transcription factor Foxp3 belong to a predominantly suppressive T-cell lineage of dual origin (11, 12). A deficiency in FOXP3 might lead to Treg cell dysfunction (13, 14). The strategies Treg cells employ to regulate T and B cell responses remain intricate and largely elusive. However, certain experiments and studies suggest that Treg cells, with their anti-inflammatory properties, might counteract the development of CVD (15, 16).

Adaptive and innate immune responses exhibit a dual role in CVD. While some immune reactions provide protective effects during the early stages of the disease, others can turn detrimental when rendered ineffective (17). Given this backdrop, the immune system emerges as an enticing target for pioneering CVD preventive therapies (18, 19). By selectively modulating this immune response related to CVD, it's plausible to devise novel treatments for the disease (20). The reduction or functional impairment of regulatory T cells (Tregs) may lead to an increase in the activity of pro-inflammatory immune cells, such as Th1 and Th17 cells, thereby enhancing the inflammatory response of the cardiovascular system. In the context of atherosclerosis, dysfunction of Tregs may exacerbate inflammation and endothelial dysfunction, accelerating the formation and progression of plaques (21). After myocardial infarction, the reduction in Tregs can lead to excessive cardiac inflammation, affecting heart repair and functional recovery. The decline in Tregs may also promote the process of cardiac fibrosis, especially in the context of certain cardiomyopathies (22). However, oxidative stress may lead to dysfunction of Tregs, particularly in environments related to cardiovascular diseases. High cholesterol levels can also affect the function and survival of Tregs. The possibility of selectively modulating protective and deleterious immune reactions in CVD may aid in a more personalized prevention and treatment regimen. For instance, LDL (low-density lipoprotein) accumulation in arterial walls is a key autoantigen in atherosclerosis (23, 24). Studies aiming to validate this concept by immunizing experimental animals with oxidized LDL particles inadvertently triggered atherogenic immunity involving regulatory T cells (25, 26).

In this review, we delve deep into the roles of Treg cells in immune modulation, their underlying mechanisms, and further investigate their impacts on atherosclerosis, myocarditis, and other cardiovascular diseases. Moreover, we spotlight and discuss experimental and clinical data on the potentiality of crafting immunotherapies to reduce cardiovascular risk. We will also recapitulate ongoing clinical studies and deliberate challenges linked to the development of effective and safe vaccines for CVD.

2 The pathogenesis of Treg cells in cardiovascular diseases

One of the primary mechanisms by which regulatory T cells (Tregs) participate in cardiovascular diseases is by modulating the immune-inflammatory responses of target T cells and antigen-presenting cells (APCs). This modulation limits T cell proliferation and cytokine production (27, 28). Tregs can regulate these immune-inflammatory responses through the release of inhibitory cytokines, the consumption of IL-2 and ATP/ADP, or through receptor-ligand interactions, such as inducing apoptosis or altering the functionality of APCs (29, 30) (Figure 1).

2.1 Production of anti-inflammatory cytokines by Tregs

Tregs play a pivotal role in the immune system by producing anti-inflammatory cytokines, such as TGF-β and IL-10. These cytokines can directly suppress other immune cells, including antigen-presenting cells (APCs) like macrophages and CD8+ effector T cells, thereby reducing inflammation and preventing excessive immune responses (31, 32).

Studies have highlighted the critical importance of TGF-β within Tregs *in vivo*, as Tregs in mice with a T-cell-specific deficiency of TGF-β failed to suppress inflammation (33, 34). Moreover, evidence suggests that Tregs can transmit membrane-bound TGF-β to corresponding receptors on effector T cells via direct cell-to-cell contact, thereby suppressing their functionality ex *vivo* (35, 36).

Similarly, IL-10 has a crucial role in Treg-mediated immune modulation, especially in response to pathogens or external stimuli-induced inflammatory reactions (37, 38).IL-10 produced by Tregs plays a key role in resisting atherosclerosis and modulating the formation of atherosclerotic plaques (39). IL-10 can prevent endothelial cell dysfunction and help maintain the health of vascular endothelial cells, protecting them from damage by inflammatory factors (40). Additionally, it reduces the expression of adhesion molecules on endothelial cells: IL-10 can decrease the expression of adhesion molecules on the surface of endothelial cells, thereby reducing the adhesion and migration of inflammatory cells (32). IL-35, another cytokine predominantly expressed in Tregs, also has a significant role in the maximal immunoregulatory functions of Tregs (41, 42).IL-35 released by Tregs promotes the differentiation of naive Tregs into mature Tregs, while mice lacking IL-35 exhibited diminished Treg suppressive functions (41). Therefore, IL-35 is also a potential target for targeting Treg cells to inhibit immune-inflammatory damage.

2.2 Depletion of IL-2 by Treg cells stimulates pro-inflammatory cytokines

IL-2 is a pivotal growth factor, crucial for T cell proliferation. Although IL-2 itself does not directly produce anti-inflammatory effects, by promoting the activity of Tregs, IL-2 can indirectly

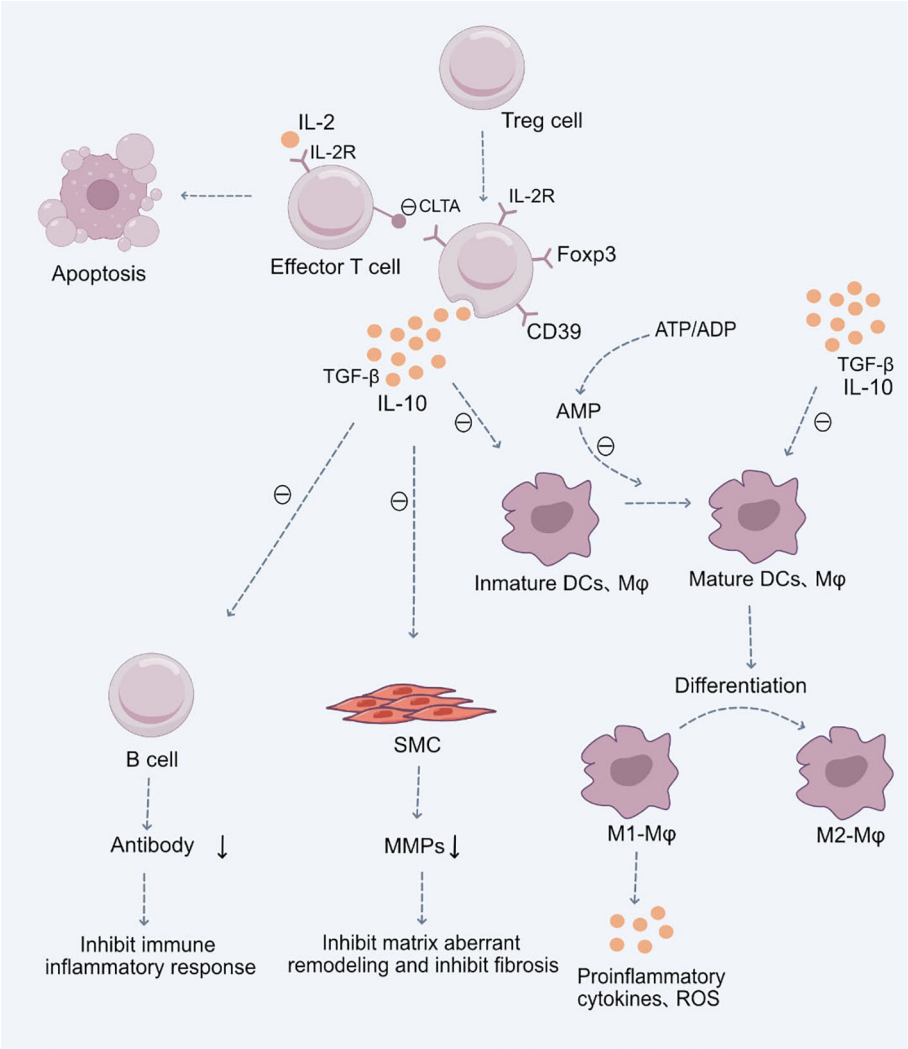

FIGURE 1
Role of Treg Cells in the Pathogenesis of Cardiovascular Diseases.Upon stimulation by antigens and cytokines, Treg cells differentiate into CD4+CD25+Foxp3+ Treg cells. These cells exert their immunosuppressive effects through the production of anti-inflammatory cytokines TGF-β and IL-10, subsequently inhibiting the functions of mature immune cells, including dendritic cells (DCs), macrophages, and effector T cells. Additionally, they suppress B cell antibody production, mitigating the immune-inflammatory response. They also inhibit the secretion of Matrix Metalloproteinases (MMPs) by smooth muscle cells (SMCs), restraining abnormal cardiovascular matrix remodeling and fibrosis. Moreover, CD4+CD25+Foxp3+ Treg cells facilitate the differentiation of pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages, thereby suppressing the production of inflammatory cytokines and reactive oxygen species (ROS). These Treg cells, with a high expression of the IL-2 receptor, competitively utilize IL-2, hindering the maturation of responding T cells and promoting their suppression and apoptosis. Certain inhibitory receptors expressed on Treg cells, such as CTLA-4, interact with ligands on antigen-presenting cells, inhibiting the latter's function and inducing their apoptosis. Lastly, the ectoenzyme CD39 expressed by Treg cells hydrolyzes ATP or ADP to AMP, reinforcing the Treg-mediated suppression of ATP-driven dendritic cell maturation.

promote the formation of an anti-inflammatory environment. The increase in Tregs helps regulate the immune response within atherosclerotic plaques, potentially aiding in reducing plaque formation and progression (43). Furthermore, by supporting the survival of Tregs, IL-2 helps maintain the balance of the immune system and immune tolerance, preventing excessive immune responses. This can help regulate the immune response within atherosclerotic plaques, potentially aiding in reducing plaque formation and progression (44). regulatory (Treg) cells possess high-affinity IL-2 receptors. By efficiently consuming IL-2, Treg cells "deplete" IL-2 from their surroundings, consequently inhibiting their own proliferation. This indirectly suppresses T cell-mediated inflammation (45, 46). IL-2 plays a critical role in the balance and development between Treg cells and effector T cells. Immune suppressive regulatory T lymphocytes expressing the transcription factor Foxp3 play an essential role in maintaining immune tolerance to self and benign non-self antigens (47). For most Tregs, differentiation requires antigenic signals from T cell receptors, costimulatory molecules, and signals from cytokine receptors like IL-2 (48, 49). Thus, by competitively utilizing IL-2, Treg cells interfere with the maturation of responsive T cells, leading to T cell apoptosis and suppression (47, 50).

Treg cells can absorb ATP and release ADP. The depletion and conversion of ATP/ADP can induce cytotoxicity and suppress the activities of nearby antigen-presenting cells (APCs) and CD8+ effector T cells (51, 52). The ectoenzyme CD39 expressed by Treg cells can

hydrolyze ATP or ADP to AMP, enhancing Treg suppression of ATP-driven dendritic cell maturation (53, 54). Additionally, the co-expression of CD39 and CD73 on Tregs can convert extracellular ADP to adenosine. Adenosine can further bind to its A2A receptor, thereby inhibiting effector T cell activities (55, 56). Notably, activation of the adenosine A2A receptor not only suppresses effector T cells but also enhances Treg function by downregulating IL-6 expression and increasing TGF-β production (57, 58).

2.3 Treg cells mediate contact inhibition of antigen-presenting cells

Certain inhibitory receptors expressed on Treg cells, such as CTLA-4, can interact with ligands on antigen-presenting cells, inhibiting their function (59, 60).

Through regulation of antigen-presenting cells, particularly dendritic cells, and macrophages, Treg cells indirectly restrict effector T cell activity (61, 62). Tregs can inhibit dendritic cell function and maturation and induce them to produce TGF-β, further suppressing effector T cell activation and differentiation (63, 64). Studies have demonstrated that, in both humans and mice, Tregs can reduce the expression of the co-stimulatory molecules CD80 and CD86 on dendritic cells (63, 65). Highly expressed cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) in Tregs plays a crucial role in this process, enhancing the regulation of dendritic cells. Some studies further indicate that Foxp3-expressing CD4^+CD25^+ regulatory T cells (Tregs) that highly express the immune checkpoint receptor CTLA-4 exhibit defects specific to Tregs, leading to severe immune inflammatory responses (63) As a critical mechanism of Treg-mediated suppression, CTLA-4 expressed by Tregs downregulates the expression of CD80/CD86 co-stimulatory molecules on antigen-presenting cells (APCs) (66, 67). Inflammation is a key factor leading to plaque instability and rupture; therefore, CTLA-4-mediated immune regulation may help increase plaque stability (68). Reducing inflammatory responses can protect vascular endothelium, preventing endothelial cell dysfunction, which is a key factor in the development of atherosclerosis and cardiovascular diseases. CTLA-4-mediated immune regulation helps control chronic inflammation, thereby aiding in the prevention of atherosclerosis progression and the occurrence of cardiovascular events (69).

Another molecule expressed in Treg cells that impacts dendritic cell function is lymphocyte-activation gene 3 (LAG3). It has a high affinity to class II molecules of the major histocompatibility complex (MHC) (70, 71). When LAG3 binds to MHC class II molecules, it activates the immunoreceptor tyrosine-based inhibitory signaling pathway, thereby reducing dendritic cell maturation and their capacity to activate T cells (72, 73).

2.4 Cytotoxic role in T-cell suppression

In T-cell suppression, cytotoxicity stands as a pivotal potential mechanism. For instance, CD8+ T cells and NK cells can directly target and kill cells through the Granzyme B and Perforin pathways (74, 75). Studies have identified that Treg cells lacking Granzyme B exhibit weakened suppressive functions. Granzyme B also plays a crucial role in the suppression of immunity by Treg cells, as it aids in the killing of NK cells and CD8+ T cells (76, 77). Granzyme B can induce apoptosis in cells within atherosclerotic plaques, such as macrophages and smooth muscle cells. An increase in cell apoptosis may lead to an increase in the cellular mass at the plaque core, affecting plaque stability. Imbalance in cell apoptosis may cause plaques to rupture more easily, thereby increasing the risk of acute cardiovascular events (76). Granzyme B-induced apoptosis of smooth muscle cells may affect the remodeling process of blood vessels, potentially impacting vascular stability and elasticity.

2.5 Treg cell-mediated suppression of B cells

Treg cells' modulation of B cell responses showcases their vital impact. Experimental evidence suggests that when Treg cells are depleted, autoantibody production increases in autoimmune mice; conversely, supplementation of Treg cells results in decreased autoantibody concentrations (78, 79). An early hypothesis postulated that Treg cells primarily modulate B cell responses by suppressing the helper T cells that assist in antibody production (80, 81). However, subsequent research indicates that Treg cells might inhibit B cells' class-switch recombination and induce apoptosis using perforin and granzymes, directly constraining antibody production (82, 83). B cells can produce pro-inflammatory antibodies, such as antibodies against oxidized low-density lipoprotein (oxLDL). These antibodies may promote inflammatory responses and the formation of atherosclerotic plaques. By inhibiting these B cells or reducing the pro-inflammatory antibodies they produce, inflammation and the progression of atherosclerosis can be mitigated (84).

2.6 Treg cells and endothelial cell interaction

Endothelial dysfunction is a key factor in the development of various cardiovascular diseases. Treg cells, by secreting anti-inflammatory cytokines such as TGF-β, can promote the integrity of the endothelial layer of blood vessels. Endothelial cells, through their membrane-bound TGFβ, convert some CD8(+) T cell populations into Treg cells. Treg cells induced by endothelial cells produce the soluble form of TGFβ1, but not TGFβ2, and they also acquire a regulatory phenotype expressing high levels of CD25 and Foxp3 (85). Vascular Endothelial Growth Factor A (VEGF-A), Interleukin 10 (IL-10), and Prostaglandin E2 (PGE2) synergistically induce the expression of FasL in endothelial cells. Due to the high expression levels of c-FLIP in Treg cells, they acquire the ability to kill effector CD8(+) T cells rather than Treg cells (86). Treg cells can reduce the expression of adhesion molecules on the surface of endothelial cells, decreasing the interaction between leukocytes and endothelial cells, thereby reducing vascular inflammation (87). Furthermore, studies have

shown that the recognition of self-antigens expressed by endothelial cells in target tissues helps in the effective recruitment of Treg cells *in vivo*. This Treg recruitment depends on the induction of MHC class II molecule expression in endothelial cells mediated by IFN-γ, and requires the activation of the T cell receptor PI3K p110δ (88). Therefore, endothelial cells and self-recognition enable the transportation of Treg cells, expanding the understanding of immune regulation dynamics, and making the interaction between vascular endothelial cells and Treg cells a potential therapeutic target for treating cardiovascular diseases.

In summary, Treg cells maintain immune homeostasis through various mechanisms, ensuring that immune responses do not exceed the necessary thresholds. Within the context of cardiovascular diseases, Treg cell function might be compromised or insufficient, leading to intensified inflammatory responses, which can accelerate the progression of ailments like atherosclerosis. As such, modulating the function and number of Treg cells might emerge as a promising strategy in treating cardiovascular diseases.

3 Pathogenic mechanisms of Treg cells in cardiovascular diseases and potential therapeutic targets:

Inflammation plays a key role in the onset and exacerbation of cardiovascular diseases (89). When inflammatory cells aggregate and release inflammatory cytokines, the progression of diseases like atherosclerosis, hypertension, and myocardial infarction can be accelerated (90, 91). Regulatory T cells (Tregs) play a pivotal role in controlling and suppressing inflammatory reactions, preserving the balance of the immune system, and preventing excessive immune responses (31, 92). A significant enhancement in inflammatory reactions is observed in the absence of Treg cells, hinting at the crucial role of Treg cells in maintaining cardiovascular health and suggesting their functionality might be influenced by their surrounding environment (93, 94). Treg cells can inhibit the aggregation of pro-inflammatory cells and the subsequent release of associated inflammatory cytokines, critical processes in cardiovascular diseases such as atherosclerosis (95). Therefore, amplifying or enhancing the function of Treg cells could present new strategies for treating cardiovascular diseases. Recognizing the role of Treg cells in cardiovascular ailments, researchers and clinicians are increasingly focusing on their potential as therapeutic targets. Modulating the number or function of Treg cells might offer novel approaches for preventing or treating inflammation-associated cardiovascular diseases (Figure 2).

3.1 Atherosclerosis

T regulatory cells (Tregs) play an indispensable role in the prevention and prognosis of atherosclerosis (96, 97). The role of regulatory T cells (Tregs) in atherosclerosis is highly diverse, including anti-inflammatory effects, maintaining immune balance, protecting vascular endothelium, and potential metabolic regulatory functions. Recent studies indicate that Tregs may also be involved in regulating metabolic pathways, such as lipid metabolism, which could significantly impact the progression of atherosclerosis (98). The ApoE-/- mouse model is extensively used in atherosclerosis research. Due to the absence of Apolipoprotein E (ApoE), these mice are prone to develop hypercholesterolemia and atherosclerosis (99, 100). Compared to normal mice, ApoE-/- mice exhibit a significant reduction in the number of Tregs (101, 102). Researchers have observed a reduced abundance of CD4+CD25+FOXP3+ Tregs in coronary artery atherosclerotic plaques, and this decrease correlates positively with the vulnerability of carotid artery plaques (103, 104) Tregs help protect vascular endothelial cells by reducing inflammation, thus preventing endothelial dysfunction. By decreasing the infiltration and activation of inflammatory cells, Tregs contribute to the stability of atherosclerotic plaques, reducing the risk of plaque rupture (105). further highlighting the protective role of Tregs further highlighting the protective role of Tregs.

Tregs are known to attenuate the accumulation of inflammatory cells, inhibit the secretion of inflammatory cytokines, and promote the transition of M1 macrophages to M2 phenotype. Consequently, this leads to a marked decrease in inflammatory cytokines and foam cells in atherosclerotic lesions (106). Anti-inflammatory cytokines released by Tregs, such as TGF-β, IL-10, and IL-35, not only enhance plaque stability but are also key in inhibiting atherosclerosis.

For instance, in the context of atherosclerosis, TGF-β exerts anti-inflammatory effects by inhibiting inflammatory cell activity, thereby preventing plaque formation and progression. Additionally, collagen, a crucial component for plaque stability, can degrade, increasing plaque vulnerability. TGF-β positively regulates collagen synthesis and deposition, aiding in maintaining vascular wall integrity (107, 108). Similarly, IL-10 also offers protection against atherosclerosis development. Absence of IL-10 exacerbates infiltration of inflammatory cells, reduces collagen content, and renders plaques more fragile. Overexpression of IL-10, on the other hand, counters these inflammatory responses and plaque formation (109, 110). In a study, induction of CD4+Foxp3+ Tregs in the spleens and aortas of ApoE-/- mice was frequently associated with significant elevations in plasma IL-35 levels (41). Further observations noted that CCR5+ Tregs in ApoE-/- exhibited a diminished AKT-mTOR signaling, elevated expression of inhibitory checkpoint receptors TIGIT and PD-1, enhanced TIGIT and PD-1 signaling, and increased IL-10 expression, all aiding in retaining the Treg immunosuppressive function (41). Therefore, IL-35 promotes the induction and differentiation of CD4+Foxp3+ Tregs, and by sustaining the suppressive mechanisms of CCR5 expanded Tregs, inhibits atherosclerosis.

B cells, pivotal cells in the immune system, primarily produce antibodies. A decrease in B cells in both ApoE-/- and LDLR-/- mice is associated with halting the progression of atherosclerosis, providing evidence of a probable pro-inflammatory role of B cells in atherosclerosis (111, 112). The exacerbation of the disease upon reintroduction of B cells further bolsters this perspective. Tregs are believed to suppress B cell activation, a suppression potentially mediated by cytokines produced by Tregs, such as IL-10 or TGF-β, or through direct cell-cell interactions (113, 114). Considering the

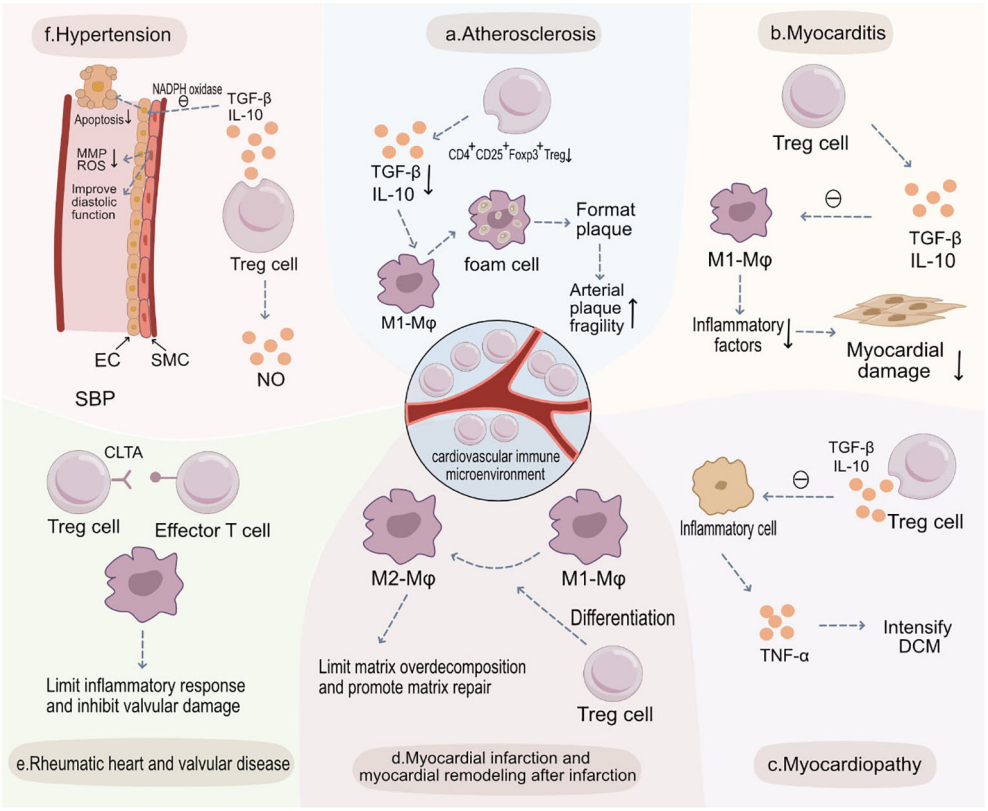

FIGURE 2
Treg Cells in the Pathogenesis of Various Cardiovascular Diseases and Potential Therapeutic Targets. **(A)** Atherosclerosis: Within atherosclerotic plaques, the abundance of CD4+CD25+Foxp3+ Treg cells diminishes, leading to a decreased release of anti-inflammatory cytokines such as TGF-β and IL-10. This results in increased engulfment of LDL by M1 macrophages and heightened foam cell formation, thus increasing plaque vulnerability. **(B)** Myocarditis: In myocarditis, CD4+CD25+Foxp3+ Treg cells release anti-inflammatory cytokines like TGF-β and IL-10, inhibiting the function of M1 macrophages, curbing immune-inflammatory reactions, and preserving myocardial tissue. **(C)** Myocardiopathy: In cardiomyopathies, there's a decrease in CD4+CD25+Foxp3+ Treg cells and secretion levels of TGF-β and IL-10, weakening their inhibitory effects on inflammatory cells. This culminates in augmented secretion of pro-inflammatory cytokines like TNF-α, exacerbating dilated cardiomyopathy (DCM). **(D)** Myocardial Infarction and Post-Infarction Remodeling: Post-myocardial infarction, CD4+CD25+Foxp3+ Treg cells aid recovery by modulating the differentiation of monocytes and macrophages into reparative M2 macrophages. They also limit post-infarction inflammation and excessive matrix degradation, thereby slowing adverse morphological changes. **(E)** Rheumatic Heart and Valvular Disease: In rheumatic heart and valvular diseases, CD4+CD25 +Foxp3+ Treg cells inhibit effector T cells and curtail sustained inflammatory responses, reducing valvular injury. **(F)** Hypertension: CD4+CD25 +Foxp3+ Treg cells, through the secretion of TGF-β and IL-10, inhibit NADPH oxidase, restoring the vasodilatory function of SMCs and decreasing endothelial cell apoptosis to preserve endothelial functionality. Additionally, they suppress the secretion of MMPs and ROS by SMCs, preventing abnormal vascular remodeling and ultimately reducing systolic blood pressure (SBP).

regulatory effects of Tregs on B cells, enhancing the function or number of Tregs might also be an effective therapeutic strategy (115, 116). While current evidence has elucidated the critical roles of B cells and Tregs in atherosclerosis, more experiments are necessary to comprehensively understand their precise roles in the disease and how to best harness this information for new therapeutic approaches.

3.2 Myocarditis and cardiomyopathy

Myocarditis, characterized by inflammation, is a cardiac disease. Following viral myocarditis, autoimmune responses may lead to sustained myocardial damage. This could be pivotal in the transition of viral myocarditis to dilated cardiomyopathy (DCM) (117, 118). When Coxsackievirus B3 (CVB3) infects cardiomyocytes, it directly damages these cells, resulting in cell death and tissue injury, an effect attributed to its direct cytotoxicity (119, 120). Moreover, the body's immune response targets the infecting virus; however, this response can occasionally be "overactive," exacerbating myocardial damage. This phenomenon is referred to as post-viral pathogenic immune responses (121, 122). While the precise etiology of myocarditis remains to be fully elucidated, current studies speculate that autoimmune responses play a crucial role in disease onset and progression.

3.2.1 Myocarditis and the role of Treg cells

Treg cells occupy a central protective role in the development of myocarditis (123).In animal models, experimental findings consistently show that natural Treg cells play a proactive role in suppressing virus-induced immunopathological responses and in preventing virus-induced tissue damage. Studies have identified a negative correlation between Treg cell abundance and the severity of myocarditis (124, 125). Thus far, numerous investigations have

highlighted the critical protective function of Treg cells in myocarditis. Prior to viral infection, Treg cells can inhibit myocarditis induced by CVB3 by suppressing pathogenic immune responses and ensuring anti-viral cardiac responses via the TGF-β-Coxsackie adenovirus receptor pathway (125, 126). Tregs suppress cardiac inflammation by secreting anti-inflammatory cytokines, such as IL-10 and TGF-β. In the context of myocarditis, this suppression helps to reduce damage to cardiac tissues (32). Additionally, the suppressive functions of Treg cells have been displayed in multiple studies, suggesting that enhancing Treg cell activity can effectively alleviate myocarditis inflammation, reduce cardiac damage, and positively influence the progression of cardiomyopathy (127, 128).

However, the relationship between immune mechanisms and cardiomyopathy may not be linear or singular. Some findings regarding the role of Treg cells in myocarditis are contradictory. Tregs maintain the balance of the immune system by inhibiting excessive immune responses, which is particularly important in preventing the development of autoimmune cardiomyopathy. In the EAM (experimental autoimmune myocarditis) model, reducing Treg cells indeed exacerbates myocarditis, consistent with traditional understandings of Treg cells (129). If Treg cells suppress the immune response against the virus, they might indirectly promote viral replication and persistence, indicating a complex negative feedback mechanism (126). For instance, in EAM model studies, reducing the number of Treg cells within the heart resulted in aggravated myocarditis in mice (130). This seems to suggest that Treg cell activation might inhibit anti-viral immune responses, facilitating viral replication and persistence, further worsening myocardial changes. Such complexity underscores the need for a holistic perspective when understanding disease mechanisms. Within the context of myocarditis and DCM, it is essential to consider not just the interactions between the virus and the host but also the interplay among various cells within the immune system.

3.2.2 Cardiomyopathy

Dilated cardiomyopathy (DCM) is a prevalent myocardial disorder characterized by ventricular dilation and diminished myocardial contractile function, which can potentially lead to heart failure (131, 132). In dilated cardiomyopathy, a major pathological feature is myocardial fibrosis. Tregs may help slow the progression of myocardial fibrosis by reducing the release of pro-fibrotic factors and inhibiting inflammation (133). Viral infections can instigate inflammatory and autoimmune responses in the myocardium, and persistent inflammation might lead to DCM (131, 134).

The TNF mouse model offers a tool to explore the relationship between immune mechanisms and DCM. TNF-α is a pro-inflammatory cytokine expressed in many inflammatory diseases (135). In the TNF mouse model, there is a heightened cardiac expression of TNF-α, leading to DCM characteristics like cardiac inflammation, ventricular dilation, and reduced ejection fraction (136, 137).

These findings suggest a potential link between immune mechanisms and the etiology and progression of DCM. Tregs may protect cardiac myocytes from damage by reducing inflammation and oxidative stress. Further research might unveil more about this disease's mechanisms and provide insights for novel therapeutic strategies. Tregs may also play a role in the neovascularization and repair of the heart, which is crucial for the recovery and functional restoration of cardiomyopathies (125). Regulatory T (Treg) cells might play a role in inhibiting the progression of myocardial inflammation in DCM (125, 138). Moreover, when CD4+T cells are depleted in mice, their myocarditis symptoms are alleviated, further confirming the pivotal role of immune mechanisms in DCM pathogenesis (139, 140).

Compared to healthy individuals, DCM patients exhibit a decline in the number and function of Treg cells. Furthermore, serum levels of TGF-β and IL-10, both associated with immune suppression, are also reduced in DCM patients. This suggests that increasing the number and enhancing the function of circulating Treg cells might be therapeutic strategies for DCM (141, 142).

In conclusion, these experimental results provide insights into the intricate relationship between dilated cardiomyopathy and immune mechanisms. Modulating immune responses, especially by enhancing the function of Treg cells, could be a promising strategy for DCM treatment. However, translating these findings to clinical applications necessitates further research. The role of Tregs in myocarditis and cardiomyopathy is vital, primarily through immune regulation, anti-fibrotic effects, protecting cardiac myocytes and vascular functions, and regulating specific immune responses. These findings offer a new perspective and potential strategies for treating heart diseases, especially in treatments targeting the immune system (138).

3.3 Myocardial infarction and post-infarction cardiac remodeling

During myocardial ischemia and subsequent myocardial infarction (MI), immune responses play a crucial role in both injury and repair (15). Cell death induced by ischemia and infarction triggers an acute inflammatory response, drawing various immune cells to the injured myocardium (143). After myocardial infarction, the heart experiences an acute inflammatory response, and Tregs alleviate inflammation-mediated damage by secreting anti-inflammatory cytokines such as IL-10 and TGF-β (144). Tregs help control the immune response following myocardial infarction, preventing additional damage caused by excessive inflammation (64). Treg cells play a pivotal role in myocardial ischemia, MI, and post-infarction cardiac remodeling (16, 145).

Activated Treg cells soon after myocardial injury, due to their anti-inflammatory properties, maintain immune-inflammatory homeostasis, aiding tissue repair. In rat MI models, elevating Treg cell numbers can prevent adverse ventricular morphological changes by reducing inflammation and directly protecting cardiomyocytes, thereby enhancing post-ischemic cardiac function (146, 147). Tregs may slow down the process of cardiac fibrosis by inhibiting the release of pro-fibrotic factors, which is crucial for the recovery of cardiac muscle function and structure (148, 149). Additionally, Treg cells support post-MI recovery by regulating the differentiation of monocytes and macrophages into

reparative M2 macrophages, diminishing post-infarction inflammation, restraining excessive matrix degradation, and thus slowing adverse morphological changes (64, 150).Accumulating Treg cells in the injured murine heart participate in regulating fibroblast behavior and function, inhibiting post-MI fibrosis, and alleviating cardiac stiffening and dysfunction (151). One study showed that Ccl17 deficiency leads to reduced left ventricular remodeling post-MI and after angiotensin II and norepinephrine administration. This was associated with diminished myocardial fibrosis, cardiomyocyte hypertrophy, and improved left ventricular contractile function (152). The evidence demonstrated that Tregs mediated the protective effects of Ccl17 deficiency against myocardial inflammation and adverse left ventricular remodeling (153). Thus, inhibiting CCL17 might be an effective strategy to promote Treg recruitment and suppress myocardial inflammation.

Research has shown that MI patients have reduced Treg cell numbers, and lower circulating TREG cells correlate with increased MI risk (154).In murine MI models, externally administered Treg cells can reduce infarct size and ischemia-induced cardiac morphological changes. Furthermore, in mice undergoing coronary artery ligation and reperfusion, the selective depletion of Treg cells exacerbates ischemia-reperfusion injury (155, 156). Furthermore, in mice undergoing coronary artery ligation and reperfusion, the selective depletion of Treg cells exacerbates ischemia-reperfusion injury (157, 158). Treg cells might play a key protective role in myocardial ischemia, MI, and post-MI cardiac remodeling by protecting the heart, inhibiting excessive inflammatory responses, promoting repair, and improving function. Hence, modulating Treg cell numbers and function might offer novel strategies for the treatment of myocardial ischemia and infarction.

3.4 Rheumatic heart disease and valvular disorders

Rheumatic Heart Disease (RHD) is a sequelae of untreated rheumatic fever, predominantly affecting the heart valves (159, 160). RHD remains a significant cause of heart failure in developing countries, especially in areas lacking timely treatment for rheumatic fever (161, 162).

After a myocardial infarction, the heart needs to restore its blood supply, and Tregs may support this process by promoting angiogenesis. Treg cells play a pivotal role in immune modulation. A decline in the number of Treg cells in the peripheral blood of patients has been observed, and this decline is more pronounced when multiple valves are affected concurrently (163, 164).In RHD, the reduction in Treg cell count might signify a compromised immunoregulatory capacity, leading to persistent inflammatory responses and valvular damage, potentially exacerbating the progression of valvular diseases (165). The simultaneous impairment of multiple valves reflects heightened inflammatory reactions or disease severity. As the disease intensifies, the immunomodulatory function of Treg cells might further diminish.

By regulating inflammation and fibrosis, Tregs may help reduce the risk of recurrence of heart disease after myocardial infarction (166). While the proportion of Treg cells may vary in RHD, there isn't a direct correlation with echocardiographically determined valvular thickness or hemodynamic alterations (164, 167). This suggests that structural and functional damages to the valves might not have a linear relationship with Treg cell quantity or proportion, implying potential interference from other factors. Treg cells possibly play a crucial role in the progression of RHD. In rheumatic heart disease, inflammation is a key factor leading to damage and dysfunction of the heart valves. Tregs can reduce inflammation of the heart valves through their anti-inflammatory action. Research and understanding of Treg cells may help us better understand the pathogenesis of rheumatic heart disease and provide new ideas and strategies for treatment (166). Tregs play an important role in the development of rheumatic heart disease and valvular diseases, especially through their immune-regulatory and anti-inflammatory functions. By controlling the inflammatory response and reducing tissue damage, Tregs help slow the progression of the disease (165). These findings offer a new perspective for the treatment of valvular diseases, especially in terms of immune-regulatory therapy.

3.5 Hypertension

Although hypertension has long been perceived as a "non-inflammatory" disease, mounting evidence highlights the central roles of the immune system and inflammation in its pathogenesis (168, 169). Chronic inflammation is considered an important factor in the development of hypertension, and Tregs may help reduce the risk of hypertension by suppressing chronic inflammatory responses (170). Chronic inflammation is considered an important factor in the development of hypertension, and Tregs may help reduce the risk of hypertension by suppressing chronic inflammatory responses (171). Activation and infiltration of T lymphocytes can be observed in numerous hypertension models, particularly within the kidneys and vessels (172). Studies suggest that T lymphocytes get activated and infiltrate target organs as blood pressure starts rising. Tregs can inhibit autoimmune reactions against vascular and renal tissues, reducing immune-mediated damage to these organs (173). The kidneys play a central role in regulating blood pressure, and Tregs may protect renal function by reducing renal inflammation and fibrosis, thereby combating hypertension (174). Cytokines and other inflammatory mediators released by these cells might promote vasoconstriction and cell proliferation, exacerbating hypertension (175). Transplantation of TREG cells can restore endothelial function.

Endothelial cells are fundamental regulators of vasoconstriction and vasodilation, and endothelial dysfunction is considered an early indicator and a trigger for hypertension, atherosclerosis, and vasculitis (176, 177).In experiments, after mice were administered aldosterone, their vascular endothelial function was compromised, evident as adverse vascular remodeling and elevated systolic blood pressure (SBP) (178). However, pre-injecting mice with TREG cells before aldosterone administration mitigated the SBP increase, thereby shielding vessels from damage (179).TREG cells might counteract vascular damage induced by angiotensin II;

transplantation of TREG cells can rejuvenate endothelial function and slow the consistent rise of SBP (180). Tregs' anti-inflammatory action may help maintain the health of the vascular endothelium and reduce endothelial dysfunction, which is crucial for the prevention of hypertension (181). The anti-inflammatory ability of Tregs can reduce inflammation of the blood vessel walls, helping to maintain normal vascular tone and blood pressure (182, 183).

Research indicates that Tregs can promote nitric oxide (NO) production, an essential endothelial relaxant crucial for appropriate vascular dilation (184). Tregs, by secreting anti-inflammatory cytokines like IL-10 and TGF-β, can inhibit the activity of other inflammatory cells, thereby conserving endothelial function (185). Additionally, studies report that TREG cells might release IL-10 to optimize microvascular endothelial function in hypertensive patients (173).IL-10 itself can reduce NADPH oxidase activity, thereby enhancing endothelial relaxation function (186). Upon transferring normal mice TREG cells into angiotensin II-treated IL-10 deficient mice, restoration of endothelial function and a decrease in SBP were observed (187).

Nevertheless, some studies present contradictory findings, suggesting that the immunosuppressive effects of transferred Treg cells ameliorate cardiac damage and improve electrical remodeling, yet the transplantation of TREG cells does not significantly influence blood pressure itself (188). The role of Tregs in hypertension and endothelial dysfunction remains under investigation, but they have been recognized as potential therapeutic targets.

3.6 Heart failure

The imbalance of the immune-inflammatory response plays a significant role in the progression of Chronic Heart Failure (CHF) (189). Immune activation and inflammation are involved in the progression of CHF, and an imbalance of Th17/Treg in CHF patients suggests that this imbalance plays a role in the pathogenesis (190). Balancing Th17/Treg may be a promising therapeutic approach for CHF patients (191). Studies have shown that catechins improve cardiac dysfunction in rats with chronic heart failure by regulating the balance between Th17 and Treg cells. Further results indicate that catechins can significantly inhibit immune activation and regulate the imbalance of IL-17/IL-10 levels (192). Therefore, catechins can reverse the abnormal polarization of TH17 and Treg in peripheral blood and spleen, improving the progression of chronic heart failure. Additionally, a key process in heart failure is cardiac fibrosis, and Treg cells slow down this process by regulating the expression of fibrosis-related cytokines (193). Anti-inflammatory cytokines produced by Treg cells, such as TGF-β and IL-10, can directly or indirectly inhibit the production of pro-fibrotic cytokines (194).

4 Potential therapeutic strategies targeting Treg cells in cardiovascular disease

In both *in vitro* and *in vivo* studies, the adoptive transfer or effective expansion of exogenous Treg cells has shown the potential to decelerate the progression of numerous cardiovascular diseases. As a result, this method is emerging as a potential therapeutic approach to cardiovascular diseases. Preliminary clinical data has reported on the efficacy and safety of targeting Treg cells through the expansion of human Treg cells ex *vivo*.

4.1 Immunomodulatory treatments:

The pro-inflammatory role of IL-1β in cardiovascular events and its interaction with the anti-inflammatory and immune-regulatory functions of Tregs constitute an important mechanism in the development of cardiovascular diseases (195). Tregs, by regulating the activity of IL-1β, can alleviate cardiac inflammation and promote healthier cardiac repair. These findings offer a new perspective for immune-regulatory treatment strategies in cardiovascular diseases (196). A randomized, double-blind trial investigated canakinumab (a therapeutic monoclonal antibody targeting IL-1β). The study, which encompassed 10,061 patients with a history of myocardial infarction, demonstrated that anti-inflammatory treatment targeting the IL-1β innate immune pathway using canakinumab significantly reduced the recurrence of cardiovascular events. When compared to the placebo group, the levels of high-sensitivity C-reactive protein in the 50mg canakinumab group showed a median reduction of 26 percentage points from baseline, 37 percentage points in the 150mg group, and 41 percentage points in the 300mg group1 (197). IL-1β may directly or indirectly affect the function and number of Tregs. For example, high levels of IL-1β may inhibit the activity of Tregs or promote the activation of inflammatory T cells, thereby impacting the inflammatory response (198). Consequently, immunomodulation targeting IL-1β offers significant protective effects against cardiovascular diseases.

IL-2 is a key factor for the survival and function of Tregs. IL-2 not only promotes the proliferation of Tregs but also maintains their suppressive function. IL-2 enhances the suppressive function of Tregs by activating specific signaling pathways, such as the STAT5 pathway (199). A randomized, double-blind, placebo-controlled phase I/II clinical trial treated patients with stable ischemic heart disease and acute coronary syndrome (LILACS) using low-dose IL-2. This trial employed Aldesleukin (a recombinant form of IL-2) to determine its safety, tolerability, and the dosage required to increase the average circulating Treg levels by at least 75% (200). Aldesleukin offers an intriguing alternative approach to achieving atheroprotective immunomodulation. The trial aimed to investigate the effects of low-dose IL treatment in augmenting Tregs. By supporting the function of Tregs, IL-2 helps reduce the inflammatory response in cardiovascular diseases, especially in conditions like atherosclerosis and myocardial infarction. Tregs, by alleviating inflammation, protect vascular endothelial function and reduce the formation of atherosclerotic plaques, thus contributing to cardiovascular health (201).

Experimental and epidemiological studies have backed the protective effects associated with various LDL-targeted antibodies (202). Apolipoprotein (apoB) is a specific lipoprotein, serving as the primary carrier of various lipids (like cholesterol) in the

bloodstream (203). It can be found in two main lipoproteins: LDL and very low-density lipoprotein (VLDL). Elevated apoB levels are correlated with an increased risk of cardiovascular disease (97, 204). ApoB may affect the function of Tregs directly or indirectly. For instance, oxidized LDL may negatively impact Tregs, reducing their suppressive ability. ApoB, by affecting Tregs, can lead to an imbalance in immune regulation, potentially exacerbating cardiovascular inflammation and the progression of atherosclerosis (97, 205). Given that each LDL and VLDL particle contains just one apoB protein, the concentration of apoB in the blood can be regarded as a representative of lipoprotein particles with potential atherogenic properties (206, 207). Building on these promising observations, Lehrer et al. tested a monoclonal antibody targeting oxidized LDL (oxLDL), MLDL1278A, for its potential to reduce inflammation in atherosclerosis (208).In the study, 147 atherosclerotic patients with inflammation in the carotid or aorta plaques were randomly divided into three groups. Results indicated that while the MLDL1278A-treated groups had higher serum concentrations, there wasn't a significant reduction in arteritis compared to the placebo group. MLDL1278A was well-tolerated, with no immunogenicity observed. In multiple dosing groups, levels of tumor necrosis factor-α and interleukin-6 showed a slight increase by the fourth week.

CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) is a crucial molecule on the surface of Treg cells. By binding to the B7 family molecules on antigen-presenting cells, CTLA-4 dependent downregulation can inhibit T-cell activation. CTLA-4 blockade triggers an over-proliferation of CD28-dependent Treg cells, and a concurrent inactivation of Treg cells is necessary for tumor rejection responses (209). Therefore, Treg cells self-regulate through a CTLA-4 and CD28-dependent feedback loop. The disruption of this loop by CTLA-4 blockade could counteract the damage caused by an overly activated immune-inflammatory response to cardiac and vascular tissues, making it a potential target for the treatment of certain types of cardiovascular diseases. Ipilimumab, an antibody drug targeting CTLA-4, is commonly used for certain types of cancer treatment, but its role in modulating Treg cells also shows potential. Treatment with Ipilimumab leads to a reduction in Treg cells mediated by macrophage ADCC, while also shifting TAM polarization from M2 to M1, subsequently attracting CD8 cells and increasing the anti-tumor response (210). GITR (Glucocorticoid-Induced TNFR-Related protein) also plays a role in the regulation of Treg cells and can influence immune responses. Some studies are exploring therapies targeting to modulate Treg cell functions (211).

4.2 Active immunotherapy using vaccines

The therapeutic use of PCSK9 antibodies has been shown to effectively lower LDL cholesterol levels, and when combined with statins, it has been proven to further reduce the risk of cardiovascular diseases (212). However, the high cost of this treatment has limited its widespread use among patients. Therefore, the potential to induce similar antibodies through vaccination is being explored as a more cost-effective approach.

An atherosclerosis vaccine based on the principle of modulating the autoimmune response against LDL-related antigens has been tested in clinical trials. This vaccine may activate or increase the number of Tregs, thereby enhancing their regulatory role in cardiovascular inflammation (99). By modulating the activity of Tregs, the vaccine could alter the immune response to LDL or oxidized LDL (oxLDL), reducing the development of atherosclerosis (213). According to experimental evidence, the primary mode of action of such vaccines is to inhibit the Th1-dependent pro-inflammatory immune response against antigens formed in modified LDL particles (110). This inhibition is mediated by antigen-specific regulatory T cells, which are activated when macrophages and other antigen-presenting cells in atherosclerotic plaques are exposed to their homologous antigens (214). Theoretically, these antigen-specific Tregs not only suppress the activity of Th1 T cells with corresponding antigen specificity but also inhibit plaque inflammation by releasing anti-inflammatory cytokines, such as IL-10 and TGF-β. The advantage of this mode of action is a lower risk of adverse side effects associated with systemic anti-inflammatory treatments. Therefore, it is expected not to lead to the slight increase in fatal infection frequency observed in the CANTOS trial (215). It has been proven in experimental models that atherosclerosis can be suppressed by activating Tregs. In the long term, a vaccine specifically inducing LDL tolerance in atherosclerotic plaques is still considered the best alternative treatment option.

A novel anti-PCSK9 vaccine formulation, termed L-IFPTA, was developed to induce the host to produce anti-PCSK9 antibodies, thereby lowering LDL-C levels in mice. This vaccine induces functional anti-PCSK9 antibodies, effectively blocking the interaction between PCSK9 and LDLR, increasing the expression of LDLR on hepatocyte surfaces, and enhancing cholesterol clearance from the bloodstream (216).The L-FPTA vaccine can also modulate the balance of the immune system, reducing levels of the pro-inflammatory factor IFN-c, and increasing levels of anti-inflammatory factors L-4 and L-10. The L-FPTA vaccine may indirectly enhance the function of Tregs by raising levels of L-10, thereby more effectively suppressing inflammation related to cardiovascular diseases. Adjusting the immune environment may impact the number and activity of Tregs, thereby altering the progression of cardiovascular diseases. The L-IFPTA vaccine significantly reduced TC, LDL-C, and VLDL-C levels in mice, diminishing the formation and severity of atherosclerotic plaques. In a preclinical study on healthy non-human primates to determine the immunogenicity and safety of the "liposomal immunogenic fusion PCSK9-tetanus toxoid adjuvant" (L-IFPTA) nanoliposome anti-PCSK9 vaccine, the data suggested that the L-IFPTA vaccine potently and safely induced these primates to produce functional anti-PCSK9 antibodies (217). This vaccine effectively stimulates a humoral immune response, generating inhibitory antibodies against plasma PCSK9, without causing systemic inflammation or adverse effects on organ functions. It also modulates the immune system balance by decreasing pro-inflammatory IFN-c levels and increasing anti-inflammatory IL-4 and IL-10 levels. The L-IFPTA vaccine demonstrates good safety and tolerability (218).

Currently, the safety of PCSK9 peptide vaccines, the ability of PCSK9 antibodies to respond and reduce LDL, has been tested in

phase I trials. Randomized placebo-controlled clinical trials evaluating anti-inflammatory drugs elucidate whether targeting inflammation itself would reduce cardiovascular events and risks. AT04A and AT06A are two AFFITOPE® peptide candidate vaccines under development, aiming to treat hypercholesterolemia by inducing specific antibodies against proprotein convertase subtilisin/kexin type 9. This phase I, single-blind, randomized, placebo-controlled study was conducted among 72 healthy participants with baseline fasting LDLc levels averaging 117.1 mg/d. Throughout the study period, the AT04A group exhibited an average reduction of -7.2% in LDL levels compared to the placebo group (219). Such antibodies help clear pathogenic particles from circulation. They can also neutralize pathogenic particles in the extracellular space, preventing their binding to pattern recognition receptors, reducing intracellular cholesterol accumulation, and inhibiting the induction of pro-inflammatory signals in macrophages. High levels of cholesterol within cells can impair the function and stability of Tregs. By reducing intracellular cholesterol accumulation, Tregs can maintain their functionality and ability to regulate the immune system effectively (99). Cholesterol metabolism is crucial for cell membrane integrity and signaling. By regulating cholesterol levels within Tregs, it's possible to influence their survival, proliferation, and capacity to suppress pro-inflammatory responses (220). Reducing cholesterol accumulation in Tregs can improve their ability to control the inflammatory processes that contribute to plaque development and stability (221).

4.3 Immune adsorption therapy

Transgenic mice expressing the Tumor Necrosis Factor-α (TNF-α) gene under the cardiomyocyte promoter (TNF1.6 mice) develop dilated cardiomyopathy (DCM). These transgenic mice exhibit widespread cardiac inflammation, suggesting that an immunopathogenic mechanism might promote cardiomyopathy. Compared to control TNF1.6 mice, TNF1.6 mice treated with monoclonal anti-CD3 or anti-CD4 antibodies displayed significant reductions in heart size and plasma troponin I concentrations due to T cell depletion. Adoptive transfer of CD4(+)CD25(+) cells from H310A1-infected mice into uninfected TNF1.6 recipients eliminated cardiomyopathy. Administration of recombinant TNF-α exogenously to H310A1-infected mice for 4 days abrogated immune suppression (222). After six months of immune adsorption therapy, the left ventricular ejection fraction in DCM patients significantly improved, correlating closely with an increase in peripheral Treg cell numbers. Experimental evidence suggests that direct transfer of Treg cells into TNF transgenic mice can reduce their heart weight and plasma levels of troponin I, alleviating DCM symptoms. Compared to healthy individuals, the number of Treg cells in the myocardium of DCM patients is notably reduced (140).In this study, induced glucocorticoid tumor necrosis factor R-related protein was used as a marker for Treg cells rather than the more specific FOXP3 transcription factor. In summary, autoimmunity modulation might be beneficial in preventing myocarditis and subsequent DCM, and manipulating the number and function of Treg cells could be a promising strategy against these challenging diseases.

5 Conclusion and prospective

While certain cardiovascular diseases such as atherosclerosis, hypertension, and myocardial infarction are not considered classical autoimmune diseases, immune responses related to self-antigens indeed play significant roles in their development (223). Specifically, T cells, especially Treg cells, occupy a central position in the pathophysiology of these diseases (224). Studies indicate that defects in the number and function of Treg cells are associated with a variety of cardiovascular diseases, and enhancing the function or number of Treg cells can effectively slow down disease progression (225). Hence, Treg cells are viewed as potential therapeutic targets. Ample experimental and clinical research suggests that a reduced number and impaired function of Treg cells might be present in multiple cardiovascular diseases (15). Adoptive transfer of exogenous Treg cells or expansion of endogenous Treg cells effectively inhibited the progression of many cardiovascular diseases (97). Although the therapeutic mechanisms of Treg cells for cardiovascular diseases remain not fully elucidated, they provide a promising research direction, potentially revealing immune-regulatory mechanisms in cardiovascular diseases.

Experimental studies have granted us many new insights into cardiovascular diseases. However, transitioning from these experimental findings to actual clinical applications often requires considerable time, given that clinical studies must consider numerous variables and complexities. In an inflammatory environment, Tregs may lose their suppressive function or transform into other types of T cells. Tregs must precisely target inflammation related to cardiovascular diseases, avoiding widespread suppression of the immune system, which could lead to infections and other adverse consequences (226). Effectively expanding and maintaining the function of Tregs *in vitro* and efficiently delivering Tregs to specific areas of cardiovascular disease present additional technical challenges. Most experimental research on cardiovascular disease vaccines focuses on early prevention. Yet, for patients in advanced stages exhibiting clinical symptoms, these study results might not be applicable. Developing methods for selectively activating or enhancing the function of Tregs, especially at sites of inflammation related to cardiovascular diseases, is important (227). Utilizing CRISPR or other gene-editing tools to enhance the stability and specificity of Tregs is another approach. Improving *in vitro* culture conditions to increase the quantity and quality of Tregs, including the use of specific cytokines and culture media, is also crucial (228). Developing new delivery systems, such as biocompatible materials and nanoparticles, can improve the stability and targeting of Tregs within the body. Combining Treg therapy with other treatment methods, such as lipid-lowering drugs or anti-inflammatory therapies, can enhance the effectiveness of the treatment (229). To translate into stable treatment regimens for cardiovascular diseases, comprehensive clinical trials are needed to understand the vaccine's mechanism of action, monitor vaccine responses, evaluate efficacy

in advanced cardiovascular diseases, and assess potential safety concerns.

Author contributions

YX: Writing – original draft, Writing – review & editing. DG: Writing – review & editing. XW: Writing – review & editing. BL: Writing – review & editing. XS: Writing – review & editing, Validation. YS: Funding acquisition, Writing – review & editing. DM: Writing – review & editing, Supervision, Conceptualization, Validation, Visualization.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Science and Technology Development Plan Project of Jilin Province, China (20210101332JC).

Acknowledgments

We would like to express our appreciation to everyone involved in drafting and preparing the manuscript.

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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 28 March 2024
DOI 10.3389/fimmu.2024.1331846

OPEN ACCESS

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RECEIVED 01 November 2023
ACCEPTED 14 February 2024
PUBLISHED 28 March 2024

CITATION
Alvarez F, Liu Z, Bay A and Piccirillo CA (2024) Deciphering the developmental trajectory of tissue-resident Foxp3$^+$ regulatory T cells. *Front. Immunol.* 15:1331846. doi: 10.3389/fimmu.2024.1331846

Deciphering the developmental trajectory of tissue-resident Foxp3$^+$ regulatory T cells

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Foxp3$^+$ T_{REG} cells have been at the focus of intense investigation for their recognized roles in preventing autoimmunity, facilitating tissue recuperation following injury, and orchestrating a tolerance to innocuous non-self-antigens. To perform these critical tasks, T_{REG} cells undergo deep epigenetic, transcriptional, and post-transcriptional changes that allow them to adapt to conditions found in tissues both at steady-state and during inflammation. The path leading T_{REG} cells to express these tissue-specialized phenotypes begins during thymic development, and is further driven by epigenetic and transcriptional modifications following TCR engagement and polarizing signals in the periphery. However, this process is highly regulated and requires T_{REG} cells to adopt strategies to avoid losing their regulatory program altogether. Here, we review the origins of tissue-resident T_{REG} cells, from their thymic and peripheral development to the transcriptional regulators involved in their tissue residency program. In addition, we discuss the distinct signalling pathways that engage the inflammatory adaptation of tissue-resident T_{REG} cells, and how they relate to their ability to recognize tissue and pathogen-derived danger signals.

KEYWORDS

Foxp3 + eT_{REG} cells, transcriptional adaptation, tissue residency, polarization, inflammation, TREG development, mucosal immunity

Abbreviations: eT_{REG}, effector T_{REG}; emT_{REG}, effector memory T_{REG}; TR-T_{REG}, tissue-resident T_{REG}; T_H, T helper cell; Foxp3, Forkhead box P3; Irf4, Interferon regulatory factor 4; BATF, Basic leucine zipper transcription factor; ST2, Suppressor of tumorigenicity 2, IL-33 receptor; TCR, T cell receptor; RORα, Retinoic acid–related orphan receptor α; Gata3, GATA-binding protein 3; T-bet, T-box transcription factor TBX21; RORγt, RAR-related orphan receptor gamma; VAT, Visceral adipose tissue; CNS, Conserved non-coding regions of the *Foxp3* locus; STAT, signal transducer and activator of transcription; mTOR, mammalian target of rapamycin; TGF-β, Transforming growth factor beta; TCF1, T cell factor 1; Icos, Inducible T cell costimulator, CD278; OXPHOS, oxidative phosphorylation; Dnmt, DNA methyltransferases.

1 Introduction

The immune system is capable of both effectively eliminating internal and external dangers and preventing exacerbated immune-mediated tissue pathology. These biological properties, coined disease resistance and disease tolerance, respectively, are complementary and require a controlled balance between pro-inflammatory and regulatory immune responses (1). This is particularly the case in mammalian hosts, where adaptive immunity allows antigen specificity to sustain long-lasting effector and memory responses that can become a potential threat to the function and homeostasis of an affected tissue long after the elimination of the danger. Amongst the mechanisms capable of controlling inflammation-generated pathology, a lymphocyte of thymic origin, a suppressor or regulatory T cell (T_{REG}), first described in the late 1960s (2), was shown to be particularly adept at immune suppression. These $CD4^+$ T cells express the Forkhead-Box P3 (Foxp3), a lineage-defining transcription factor that governs a large part of their transcriptional program through the repression of pro-inflammatory genes (e.g. *Il2, Ifng*) and the activation of genes essential for their suppressive functions (e.g. *Il2ra* (CD25), *Ctla4, Lag3, Entpd1* (CD39), *Nt5a* (CD73), *Il10, Tgfb1, Gzmb*) (3, 4). In addition, some key signature genes are prominently expressed by these cells when compared to conventional T cells, including *Ikzf2* (Helios), *Tnfrsf18* (GITR), *Nrp1* (Neuropilin 1), and *Itgae* (CD103) (5). In their capacity, T_{REG} cells occupy a central position in the immune response, and are required to ensure tolerance to self-antigens (6, 7), innocuous allergens (8, 9), and commensal microflora (10), promote tissue function and regeneration (11), and prevent and control immunopathology (12).

In a mature immune system, T_{REG} cells isolated from tissues encompass a pool of antigen-experienced $CD45RA^-CD69^+$ $CD45RO^+$ cells that differ in developmental origin, possess unique functions, and display distinct stages of activation (13). A prominent population of T_{REG} cells found in all organs are tissue-resident T_{REG} (TR-T_{REG}) cells that differ from effector memory T_{REG} cells (emT_{REG}) in that they display higher levels of the alpha E integrin (CD103) (14), lose CCR7 expression, and lose the ability to re-circulate to lymphoid organs (15). Despite the lack of a consensus on the markers to distinguish TR-T_{REG} and emT_{REG} cells in tissues, recent studies have been able to capture the high degree of transcriptional and post-transcriptional modifications that "precursor" TR-T_{REG} cells acquire to localize to non-lymphoid organs, survive, and adjust their specialized functions *in situ* amidst unfavorable inflammatory, osmotic, or metabolic conditions (16). This program involves the expression of a set of core genes that are typically upregulated, albeit at different levels, by TR-T_{REG} isolated from distinct organs, including the expression of the IL-33 receptor ST2 (17), RORα (18, 19), Icos (20, 21) and Gata3 (22–24). Amongst these differentially expressed proteins, ST2 was recently proposed to distinguish TR-T_{REG} from emT_{REG} (17). Moreover, while there is evidence TR-T_{REG} cells seed non-lymphoid organs, such as the lungs, as early as 8 days of life (25, 26), other TR-T_{REG} cells, like visceral adipose tissue T_{REG} (VAT-T_{REG}), accumulate progressively with age (27), suggesting a highly dynamic and developmental path that is largely organ-specific. Critically, there is novel evidence on the developmental trajectory that lead TR-T_{REG} cells to fully establish in the tissue. For example, recent evidence highlights how the TCR repertoire is a central determinant of TR-T_{REG} localisation (16, 28).

Currently, much remains to be understood regarding the origin of TR-T_{REG} cells. Can TR-T_{REG} cells be generated from emT_{REG} cells after the resolution of inflammatory events (29), or do they constitute stable and distinct populations of T_{REG} cells? Seeing that T_{REG} cells found in tissues can originate from the thymic selection process (thymic-derived; tT_{REG}) or be generated from the induction of *Foxp3* in naïve $CD4^+$ T cells in the periphery (peripherally-induced; pT_{REG}), can both subsets be considered TR-T_{REG} cells? Thus, a better understanding of the origin, function, and fate of TR-T_{REG} cells is required before we can harness their therapeutic potential.

In this review, we describe the steps required for the generation of TR-T_{REG}, starting from thymic selection and spanning to TCR engagement in the periphery, the switch to distinct metabolic strategies, and the modulation of Foxp3 expression that enables the adoption of key epigenetic and transcriptional changes, which, in turn, lead to the expression of a program that is highly adapted to the target tissue (Figure 1). These processes involve signaling pathways that can, when in excess, hinder, either temporarily or permanently, the stability of their core transcriptional program, revealing mechanisms by which local inflammation guides the timing and potency of immune suppression. Finally, we attempt to guide the reader through the unique signaling events that can lead tissue-resident T_{REG} cells to control type 1, type 2, and type 3-driven inflammation.

2 Origin of tissue-resident T_{REG} cells

Commitment of the T_{REG} cell lineage can occur at various stages of the T cell life cycle. During their development in the thymus, immature thymocytes are selected for the establishment of a functional TCR repertoire. Subsequently, self-reactive thymocytes are either clonally deleted or diverged into a regulatory cell fate as part of a process known as central tolerance. Despite this, a very small fraction of thymocytes escape central tolerance stochastically and must be kept in check by self-reactive thymic-derived T_{REG} cells (tT_{REG}), making them critical mediators of peripheral tolerance. Importantly, the events giving rise to tT_{REG} cells require optimal TCR signals and a unique combination of cytokines. However, the peptide pool to which thymocytes are exposed to during this selection process does not ensure complete tolerance towards innocuous non-self-antigens such as commensal bacterial peptides or allergens.

This type of peripheral tolerance often requires the *in situ* induction of peripheral T_{REG} cells (pT_{REG}) that possess unique non-self TCR repertoires (30–32) and confer them with non-redundant roles in maintaining homeostatic conditions at barrier sites like the lung and colon. In adoptive transfer models, pT_{REG} cells are capable of suppressing local inflammation in both the colon and the lungs (32–34), but are less efficient at suppressing systemic inflammation

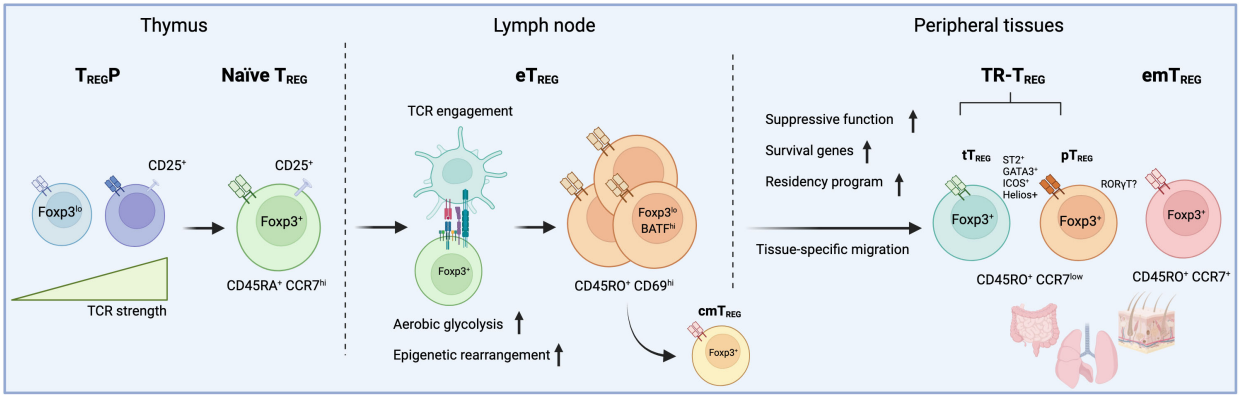

FIGURE 1
The developmental trajectory of tissue-resident T_{REG} cells involves a series of events starting from thymic selection to peripheral TCR engagement. In this figure, the trajectory of peripheral regulatory T (T_{REG}) cells is depicted, as currently defined by recent multi-omics approaches conducted in various lymphoid and non-lymphoid tissues. During thymic selection, precursor regulatory T cells ($T_{REG}P$) expressing self-reactive T-cell receptors (TCR) give rise to a pool of naive CD45RA$^+$CCR7^{hi} regulatory T cells (T_{REG}). Once in circulation, these T_{REG} cells encounter their specific antigen, triggering an activation cascade that results in a metabolic shift and chromatin remodeling. Subsequently, CD45RO$^+$CD69^{hi} effector regulatory T cells (eT_{REG}) can either stay in lymph nodes as central memory (cmT_{REG}) or migrate to tissues, where they become tissue-resident (TR-T_{REG}) or effector memory regulatory T cells (emT_{REG}). While thymic-derived TR-T_{REG} cells comprise a large portion of T_{REG} cells in tissues, T_{REG} located in the gut, for example, include peripherally-induced regulatory T cells (pT_{REG}). The absence of clear markers poses a challenge in distinguishing between these two populations *in situ*. In addition, while TR-T_{REG} cells isolated from various tissues typically display a conserved phenotype marked by the expression of ICOS, ST2, Helios, and GATA3, a significant portion of T_{REG} cells in the gut exhibit a distinctive RORγT-driven phenotype. Interestingly, there is cumulating evidence that T_{REG} cells lacking Helios expression may be more driven to express RORγT, suggesting a possible segregation between TR-T_{REG} cells derived from the thymus or induced in the periphery.

(31). Indeed, the distinct transcriptional profiles of tT_{REG} and pT_{REG} cells indicate they favour different suppressive mechanisms that vary in effectiveness in a context-dependent manner (31). Yet, despite these potential differences, attempts at identifying markers in pT_{REG} cells that are distinct from tT_{REG} have so far failed (35, 36), rendering them mostly undistinguishable at barrier sites. While Helios and Neuropilin 1 (Nrp1) are highly expressed by tT_{REG} cells (30, 37), neither Helios (36) nor Nrp1 (38), were found to be exclusively expressed by these cells. Thus, despite their distinct origin, TCR repertoire, and functions, pT_{REG} cells cannot be distinguished from the pool of tT_{REG} cells in mucosal tissues, and further investigation into features that demarcate each subset is warranted.

2.1 Thymic development of T_{REG} cells

Thymic-derived T_{REG} cells undergo the same early core processes of thymic selection as conventional CD4$^+$ T cells (39, 40). Namely, newly seeded thymocytes undergo V(D)J recombination in the thymic cortex to generate productive TCR chains capable of self-MHC recognition. Upon successful TCR signaling, committed thymocytes migrate into the thymic medulla where they encounter medullary thymic epithelial cells (mTECs) that express promiscuous transcription factors AIRE and Fezf2, allowing them to transcribe and present tissue-restricted antigens (TRAs) to developing thymocytes (41, 42). Here, thymocytes that are strongly reactive toward TRAs and other self-antigens are deleted, while weaker stimulation and the presence of certain cytokines such as TGF-β and IL-2 can skew their fate toward T_{REG} cell differentiation (43–47).

Optimal TCR signaling is the predominant factor driving T_{REG} cell lineage commitment in the thymus. TCR:peptide-MHC interaction triggers a series of phosphorylation events resulting in downstream activation of NFAT, AP-1, and NF-κB family transcription factors (48, 49). Together, these events lead to different T cell lineage specification in the thymus, as well as T cell survival, expansion, and effector function in the periphery. Expression level of the orphan nuclear receptor Nur77 (*Nr4a1*) has been directly linked to TCR signaling strength, and its expression level is elevated in T_{REG} cells compared to conventional T cells in a TCR-dependent manner (50). Unsurprisingly, since co-stimulatory molecules such as CD28 profoundly augment TCR signaling strength via NF-κB activation, they were found to play an essential role in tT_{REG} cell differentiation (46, 51–53). *Foxp3* transcription is intricately regulated by transcription factor complexes binding at its promoter and four conserved noncoding sequences (CNS), termed CNS0 to CNS3. Upon TCR stimulation, downstream activation of the NF-κB pathway results in the recruitment of c-Rel to the *Foxp3* locus at CNS3, which acts as a *Foxp3* transcriptional enhancer that is responsive to TCR signaling alone (54, 55). By dissecting each CNS region through targeted mutations, Zheng and colleagues demonstrated that CNS3 is the region that acts as a pioneer element to the generation of tT_{REG} cells, while CNS1, a region known to bind TGF-β-induced SMAD factors, and CNS2, a region targeted by CREB and STAT5 signals, were not essential to the induction of Foxp3 in tT_{REG} precursors (55), which still require cytokine signaling to become mature and functional tT_{REG} cells (43–47).

Cytokines, particularly common γ (γc) cytokines, are critical for T_{REG} cell development. IL-2 is known to be essential for commitment to the T_{REG} cell lineage (56, 57), as well as its maintenance (58). IL-2 signaling mediates STAT5 binding to the distal enhancer CNS0 as well as the promoter of *Foxp3* (56, 59), and sustains the constitutive expression of Foxp3 through CNS2 binding (57, 59). Not only does STAT5 directly facilitate Foxp3 transcription, Foxp3 also binds to the IL-2 receptor alpha chain (IL2Rα) as a transcriptional activator (60). Completion of this feedforward loop via paracrine IL-2 signaling is obligatory for T_{REG} cell development and homeostasis. Other STAT5-activating γc cytokines have also been linked to T_{REG} cell development, albeit mostly as a compensatory mechanism for impaired IL-2 signaling (45). In addition, TGF-β has also been implicated in tTreg development. While either of its downstream transcription factors, SMAD2 or SMAD3, can directly regulate Foxp3 transcription (61, 62), deletion of the SMAD binding site in the *Foxp3* locus predominantly affects the induction of pT_{REG}, but not tT_{REG} cells (62, 63). Yet, deletion of the TGF-β receptor TβRI during thymocyte development results in severely reduced T_{REG} cell numbers and defective T_{REG} cell function (64). Nonetheless, a recent study might reconcile the paradoxical discoveries. SMAD3/4 can trigger a PKA-dependent signaling cascade that causes the cessation of TCR signaling (65). Thus, the role of TGF-β in tT_{REG} differentiation could most likely be attributed to its effects on TCR signaling rather than direct transcriptional regulation of Foxp3.

2.2 The role for thymic selection events in the genesis of tT_{REG} and pT_{REG} cells.

In recent years, accumulating evidence shows that the nature of TCR signaling during thymic selection influences T_{REG} cell response to signals long after thymus egress. Notably, TCR engagement during thymic selection is a critical step in the establishment of a CpG hypomethylation pattern that characterises the epigenetic background of tT_{REG} cells (66). Numerous studies have identified two distinct tT_{REG} precursor ($T_{REG}P$) populations thought to develop into $CD25^+Foxp3^+$ tT_{REG} cells (47, 67–69). The more common $CD25^-$ $Foxp3^{low}$ and less abundant $CD25^+$ $Foxp3^-$ $T_{REG}P$ cells were shown to have distinct TCR repertoires with affinity to auto-antigens (67). In the thymus, the two $T_{REG}P$ populations display different cytokine and TCR-signaling requirements (47). Importantly, $CD25^+$ $T_{REG}P$-derived T_{REG} cells are specifically capable of suppressing experimental autoimmune encephalitis (EAE), whereas $Foxp3^{low}$ $T_{REG}P$-derived T_{REG} cells cannot (67), suggesting a functional bias within the T_{REG} population. For example, murine T_{REG} cells from the colonic lamina propria that express the same TCRα/β sequence have related transcriptional programs (70), illustrating the close relationship between TCR and the transcriptional fate of antigen-experienced memory T_{REG} cells.

Interestingly, while the relationship between TCR specificity and the establishment of TR-T_{REG} cells is not entirely understood, there are experimental examples that suggest the TCR repertoire generated during thymic selection is critical to the destination of both tT_{REG} and naïve T cells. For example, T_{REG} cells transgenic for a VAT-T_{REG}-derived TCRα/TCRβ gene arrangement will preferentially migrate to adipose tissue and differentiate into VAT T_{REG} cells (28). Yet, while these observations suggest TR-T_{REG} cells possess a largely self-specific TCR repertoire, earlier work in viral infection mouse models demonstrated that antigen-experienced T_{REG} cells with predominantly non-self TCR repertoires are generated during tissue injury and activate during re-infection (13, 71), suggesting they also contribute to the TR-T_{REG} pool. In addition, in transgenic mice possessing a fixed TCR-β sequence isolated from a $Foxp3^+RORγT^+$ colonic T_{REG} cell, T cells upregulate Foxp3 in the mesenteric lymph node prior to expressing RORγT in the colon (72). As such, both self and non-self-reactive TCR repertoires are key drivers in the generation of TR-T_{REG} cells.

2.3 The role of IL-2 and TGF-β

While the strength of TCR signaling acts as the predominant driving force for tT_{REG} cell differentiation, cytokines play a more influential role in the periphery both in maintaining tT_{REG} homeostasis and generating pT_{REG} cells. The signals that lead to the generation of pT_{REG} cells involve chronic suboptimal TCR signaling (73–75) and cytokines such as TGF-β and IL-2 to generate Foxp3-expressing T_{REG} cells *in vitro* (76, 77) and in tissues (78–80). In addition, TGF-β has been shown to strongly promote Foxp3 induction through its downstream transcription factors (SMAD2 or SMAD3) that target CNS1 (61). Consequently, a deletion of CNS1 predominantly affects the induction of pT_{REG}, but not tT_{REG} cells (63). Lastly, pT_{REG} cell induction via TGF-β can be further augmented by DC-derived retinoic acid in the lamina propria as well as short chain fatty acid metabolites of commensal bacteria (81, 82), ensuring the establishment of tolerance at mucosal surfaces. While these examples of signals that promote pT_{REG} induction are part of a complex signaling system that merits its own review, they share the common outcome of facilitating *Foxp3* expression in tissue-resident T cells, and further the importance of this transcription factor in forming the regulatory program of tissue-resident $CCR7^{low}CD69^+CD45RO^+$ T_{REG} cells.

3 The epigenetic and transcriptional trajectory of T_{REG} cells

The factors that regulate the differentiation of TR-T_{REG} remain to be fully understood. Miragaia and colleagues demonstrated through single-cell RNA-seq analysis of lymphoid and non-lymphoid (colon and skin) T_{REG} cells that these tissue-specific adaptations originate from events happening in their respective draining lymph node (19). By tracing TCR clonotypes from draining lymph nodes to their respective tissue, the authors were able to establish a pseudo-space relationship detailing the series of events that drive the generation of specialized T_{REG} cells. They were able to establish that T_{REG} cells are activated, switch to a glycolytic metabolism, and cycle rapidly prior to acquiring genes involved in migration to the tissue (19), revealing conserved stages involved in

the generation of TR-T_{REG} cells. As such, this seminal work provided confirmation that progressive transcriptional changes guide the generation of eT_{REG} cells that become TR-T_{REG} cells and highlighted how, despite tissue-specific differences, these cells share a series of epigenetic modifications that allow them to migrate, survive, and function at specific non-lymphoid sites.

3.1 The importance of peripheral TCR engagement in the generation of TR-T_{REG} cells

The engagement of the TCR of naïve T_{REG} cells is an important prerequisite for the development of tissue-specialized T_{REG} cells (83, 84), as it promotes a signaling cascade that elicits the expression of key regulatory genes leading to the suppressive activity of T_{REG} cells (85). Additionally, TCR engagement can induce epigenetic and transcriptional changes in T_{REG} cells, some of which are directly influenced by Foxp3, while others act independently (66). People affected by a loss-of-function mutations in STIM1 or ORAI1, proteins involved in store-operated calcium entry (SOCE), encounter a loss of peripheral tolerance despite some cases displaying normal T_{REG} numbers in circulation (86, 87). Similarly, impairing the normal Ca^{2+} influx during TCR engagement by deleting proteins that form the Ca^{2+} release-activated Ca^{2+} (CRAC) channels (STIM1 and STIM2) in mice specifically prevents the differentiation of activated T_{REG} cells into follicular and tissue-resident memory T_{REG} cells and generates a cascade of inflammation leading to multiorgan autoimmunity (88).

3.2 Aerobic glycolysis in the activation and clonal expansion of T_{REG} cells

Another critical factor involved in the differentiation and clonal expansion of activated T_{REG} cells is the adoption of aerobic glycolysis. This was notably demonstrated in the skin, as aerobic glycolysis by activated T_{REG} cells is required prior to their migration (89). This may, at first glance, seem counter-intuitive, as there is ample evidence that mature T_{REG} cells adopt fatty-acid oxidation (FAO) as a critical metabolic strategy to survive and suppress immune responses in tissues (90). Yet, while less efficient than oxidative phosphorylation (OXPHOS), adopting aerobic glycolysis is a critical step that occurs during T cell activation by rapidly providing the needed energy for expansion and migration, all-the-while maintaining fatty acid and amino acid reserves for cell division and protein synthesis (91). This is further evidenced by the fact that the mammalian target of rapamycin 1 (mTORC1) which is required for aerobic glycolysis, is not necessary for the thymic or peripheral development of T_{REG} cells, but essential to the function and activation of T_{REG} cells (92). Indeed, to avoid losing their suppressive program, T_{REG} cells balance the intensity of the mTORC1 and mTORC2 pathways (93), a process that is critical during their differentiation. Importantly, however, increasing glycolytic metabolism in T_{REG} cells temporarily deprives them of their suppressive capacity (90, 94), providing further evidence that the differentiation and clonal expansion of T_{REG} cells is contained within a short window of time. As such, the maturational process leading T_{REG} to become eT_{REG} cells requires both TCR engagement and a shift in their metabolic strategy (Figure 1).

3.3 The role of Foxp3 in the specialization of memory T_{REG} cells

The Foxp3-driven transcriptome of T_{REG} cells is comprised of a T_{REG}-specific gene signature and a gene set associated with an activation program which is shared with conventional T cells (95). A lymphoproliferative pathology had been previously observed in "Scurfy" mice where the X-linked *Foxp3* gene encountered a frame-shift mutation that completely disrupts the transcription of Foxp3 (96), confirming the key role of Foxp3 in establishing the suppressive program of T_{REG} cells. Point mutations in *Foxp3* that interfere with its function are the cause of a frequently fatal pediatric hereditary syndrome called immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome (97), featuring early onset diabetes, severe diarrhea, and eczema, which highly reflects the pathology of 'Scurfy" mice. Restoring Foxp3 transcription in mice whose T_{REG} cells were genetically engineered to block Foxp3 expression rescues them from severe autoimmunity as it effectively reinstates their suppressive function (12). However, while Foxp3 is essential for the establishment of T_{REG} cells, it does not determine, by itself, the entire epigenetic and transcriptional identity of mature T_{REG} cells (5, 98, 99). Rather, Foxp3 ensures that inflammatory and non-inflammatory signals encountered in the periphery do not destabilise the core suppressive program of T_{REG} cells (98, 100).

Evidence for the unique roles of Foxp3 in non-lymphoid tissues comes from the observation that functional single nucleotide polymorphisms (SNPs) in the human *Foxp3* gene do not generate a homogeneous pathology (97), with multiple accounts of IPEX-related mutations having distinct functional consequences on T_{REG} cells (101). By transposing human-isolated *Foxp3* mutations in conserved murine *Foxp3* motifs, Leon and colleagues confirmed that spontaneous multiorgan autoimmunity is largely attributed to mutations in the DNA-binding motifs, while mutations outside these motifs, notably in the N-terminal regions, lead to organ-specific dysregulation of T_{REG} cell function (101). In particular, a K199del mutation situated in the zing-finger (ZF) domain or mutations R51Q or C168Y in the N-terminal regions are prone to generating symptoms of enteropathy and skin disorders, while a R337Q mutation in the DNA-binding Fork-head domain can, in addition to these symptoms, lead to the development of diabetes mellitus (101). In addition, a murine model mimicking an A384 mutation in *Foxp3* was shown to specifically impair T_{REG} cell function in the periphery, directly impairing the ability of Foxp3 to recognize target genes and altering BATF expression (102), a key transcription factor required for TR-T_{REG} generation (103). As such, the ability of Foxp3 to interact with multiple partners is required to preserve the functional integrity of T_{REG} cells in peripheral tissues.

Although there are elements that suggest protein-protein interactions are critical to this process, we are currently limited in our understanding of how the different molecular complexes that partner with the N-terminal region of Foxp3 (104, 105), such as Tip60, Hdac7, Hdac9, Gata3, c-Rel, Foxp3, Runx1 or Eos, influence the specialization of T_{REG} cells. This is imparted by the fact that it is particularly difficult to dissociate their functions during the early events leading to the differentiation of these cells and the events that happen later in the tissues. One such example is the interaction of Foxp3 with the chromatin remodeling transcription factors TCF1 (encoded by *Tcf7*) and lymphoid enhancer binding factor 1 (Lef1) of the high-mobility group (HMG) family. In mice, the combined knock-out of both *Tcf7* and *Lef1* (*Foxp3*^{CRE}*Tcf7*$^{fl/fl}$*Lef1*$^{fl/fl}$) does not perturb lymphoid T_{REG} cells but hinders the capacity of colonic T_{REG} cells to suppress DSS-mediated colitis (106). Mechanistically, the molecular complexes TCF1 and Lef1 form with Foxp3 allow T_{REG} cells to control inflammation by repressing genes associated with excessive cycling and cytotoxic function (*GzmB*, *Prf1, Ifng*) and promoting genes associated to a T_{REG} suppressive program (106). Bulk RNAseq of murine mesenteric T_{REG} cells deficient in TCF1 (*Foxp3*^{CRE}*Tcf7*$^{fl/fl}$) show enhanced expression of core genes (including *Il2ra*, *Foxp3*, *Tgfb1* and *Lef1*), and a concomitant increase in both pro-inflammatory genes (including *Il6ra*, *Ifngr2*, *Stat3*) and genes involved in TCR activity compared to T_{REG} cells from control mice (107). These data suggest that TCF1 helps maintain a core T_{REG} program and suppress the expression of pro-inflammatory genes during TCR engagement. Similarly, Lef1 is part of an activated T_{REG} program (108), and *in vitro* gain-of-function experiments reveal it reinforces the expression of Foxp3 target genes (108). As such, these observations indicate that when Foxp3 is abundantly expressed, it interacts with both TFs to suppress pro-inflammatory gene expression and reinforce its own transcriptional profile (109). Yet, both murine and human activated (CD45RO$^+$) T_{REG} cells display lower *Tcf7* and *Lef1* expression than conventional T cells (T_{CONV}) (110) as Foxp3 directly suppresses *Tcf7* transcription and protein production, and reduces chromatin accessibility in regions targeted by TCF1 (95). As such, the highly-regulated chromatin-remodelling effect of TCF1 and Lef1 on T_{REG} cells are likely required for their further differentiation and effector function. Furthermore, pseudo-time analysis from single cell RNA-seq data of lymphoid and non-lymphoid activated T_{REG} cells reveals *Tcf7* and *Lef1* to be particularly expressed by lymphoid T_{REG} cells prior to their tissue migration (19), reinforcing the notion that TCF1 and Lef1 are involved during the early specialization events of T_{REG} cells. For example, a T_{REG}-specific depletion of *Lef1* abolishes the generation of follicular T_{REG} (T_{FR}) (107), suggesting Lef1 promotes the generation of these cells in a process similar to what is observed in follicular helper T cells (T_{FH}) (111). In addition, when compared to murine activated TCF1$^-$ T_{REG} cells, TCF1$^+$ T_{REG} cells display higher mRNA expression of transcription factors associated to helper T cells, including *Gata3*, *Tbx21* and *Rorc* (107). Collectively, these examples highlight how changes in chromatin accessibility in T_{REG} cells happen mostly after TCR engagement in the lymph node. Nonetheless, Lef1 and TCF1 are but a part of a wide network of known Foxp3-binding partners (104) whose role in defining the specialisation of T_{REG} cells remain ill-defined.

3.4 Epigenetic control of T_{REG} differentiation

To effectively reach the tissue, T_{REG} must undergo a series of epigenetic and transcriptional changes that ensure chromatin accessibility in key genes (112). Interestingly, direct comparison between human and murine T_{REG} cells reveal evolutionarily conserved epigenetic mechanisms involved in defining a T_{REG} cell program (110). Histone methylation is an important component in the processes that govern DNA accessibility and, ultimately, a T_{REG} cell transcriptional signature. Importantly, while T_{REG} cells undergo a series of chromatin remodeling events, they actively maintain CpG motif demethylation within the intronic enhancer CNS2 of the *Foxp3* locus (55, 113, 114). Maintaining an open chromatin structure in the CNS2 allows for the robust transcription of *Foxp3* by multi-molecular complexes including Foxp3 itself, NFAT, c-Rel, STAT5, Runx1-CBFβ, CREB/ATFx and Ets1 (114–118). Incidentally, a loss of any of these transcription factors or the methylation of CNS2 impairs the transcription of *Foxp3* and, ultimately, the suppressive function of T_{REG} cells in the periphery (114–119), confirming that Foxp3 is critical for the stability of the transcriptional program of tissue-localised T_{REG} cells.

Tagmentation-based whole-genome bisulfide sequencing of lymph node and tissue-isolated murine T_{REG} reveals these cells undergo multiple rounds of DNA alterations before adopting a tissue-residency program, with up to 4000 genes involved in either gain or loss of methylation (120). The processes that govern the establishment of a T_{REG} program by histone modifications have been elegantly reviewed by Joudi and colleagues (121). Globally, a delicate balance between DNA methyltransferases (DNMTs), ten-eleven translocation dioxygenases (TET), histone acetyltransferases (HATs), and histone deacetylases (HDAC) govern the stability of the T_{REG} cell transcriptional program (119, 121), but can be directly influenced by polarizing signals provided during TCR engagement.

Methylation of cytosines located in CpG-rich regions are largely governed by Dnmt1, Dnmt3a and Dnmt3b (122, 123). Interestingly, the conditional deletion of Dnmt1, but not Dnmt3a, in murine T_{REG} cells causes a loss of peripheral tolerance by 3 to 4 weeks of life, yet the cells maintain their expression of Foxp3 (124). However, these T_{REG} cells display enhanced expression of pro-inflammatory cytokines (IFNγ, IL-6, IL-12, IL-17, IL-22), chemokine receptors (CCR1, CXCR6), and transcription factors (Runx2, Stat3), highlighting the role of Dnmt1 as a non-redundant epigenetic silencer (124). During the S phase, Dnmt1 acts in partnership with the epigenetic regulator ubiquitin-like with plant homeodomain and RING finger domains 1 (Uhrf1) to govern the suppression of these gene loci (125, 126), making both Dnmt1 and Uhrf1 important therapeutical targets for the control of T_{REG} stability and function. Yet, because of the necessity of T_{REG} cells to acquire a set of genes associated with pro-inflammatory T cells, it remains to be understood how both regulators act during T_{REG} cell generation. For example, pharmacological inhibition of PI3K through its PIP4K-associated kinase results in a specific decrease in Uhrf1 in human T_{REG} cells but not TCONV cells (127), suggesting that the strength of TCR signaling plays a role in the way T_{REG} cells govern DNA accessibility of pro-inflammatory

genes. In addition, signaling by TGF-β leads to the phosphorylation and subsequent sequestration of Uhrf1 outside the nucleus (128), possibly preventing its partnering with Dnmt1.

On the other hand, the modulation of histone acetylation and deacetylation on the epigenetic adaptation of T_{REG} cells remains ill-defined. Foxp3$^+$ T_{REG} cells have been found to express histone acetyltransferases (HAT), including p300, Tip60 and CBP, as well as most members of the histone deacetylase family (HDAC) (129). Pan-HDAC inhibitors, for example, promote the acetylation of *Foxp3* and the suppressive functions of T_{REG} cells (130), confirming the importance of regulating histone acetylation to maintain a T_{REG} transcriptional program. Interestingly, HATs and HDACs are clearly involved in the helper differentiation of T_{CONV} cells (131), and further investigation is required to understand how they govern the differentiation of T_{REG} cells.

3.5 The roles of BATF and Irf4 in the generation of TR-T_{REG} cells

During these early differentiating steps, some transcriptional regulators are found to be particularly critical for the generation of TR-T_{REG} over other emT_{REG} subsets. At its core, the acquisition of a tissue residency program of TR-T_{REG} cells is closely matched to the expression of basic leucine zipper ATF-like transcription factor (BATF) and its downstream targets (16). Delacher and colleagues identified a BATF-dependent transcriptional program that drives, notably, the expression of the IL-33 receptor ST2 (120), a receptor specifically found in TR-T_{REG} (17). A T_{REG}-specific BATF deficiency in mice (*Foxp3*^{CRE}*Batf*$^{fl/fl}$; BATF$^{-/-}$), results in a multiorgan autoimmune disease with death initiating at 6 weeks of age (103). BATF$^{-/-}$ T_{REG} cells fail to accumulate in the lungs, colon, liver, and spleen, and display reduced chromatin accessibility to genes involved in T_{REG} survival in tissue, including *Gata3*, *Irf4*, *Ikzf4*, *Ets1* and *Icos* (103). In addition, *Foxp3*^{CRE}*Batf*$^{fl/fl}$ mice generate exT_{REG} cells that lose T_{REG}-associated genes (*Ctla4, Tgfb1, Foxp3*) and adopt inflammatory genes (*Rorc, Il6ra, Stat3*) (103). Specifically, ATAC-seq of murine BATF^{WT} and BATF$^{-/-}$ T_{REG} cells reveals BATF acts as a chromatin regulator, facilitating the expression of TR-T_{REG}-associated genes, including *Ctla4*, *Icos*, *Gata3*, and *Irf4*, and preserving the demethylated state of the CNS2 region of *Foxp3* (103), positioning BATF as the epigenetic guardian of T_{REG} cells as they undergo their differentiation into specialized memory T_{REG} cells.

Another transcription factor (TF) observed to be highly expressed by T_{REG} cells following TCR engagement is the interferon regulatory factor 4 (Irf4) (132). Foxp3 can directly promote the transcription of *Irf4* (133) and the BATF-JUN complex (134). In turn, Irf4 collaborates with BATF to further promote T_{REG} activation, proliferation, and transcriptional differentiation (135). Ding and colleagues demonstrated that upon TCR engagement, T_{REG} cells express the SUMO-conjugating enzyme UBC9 to specifically stabilise Irf4 function (136). While not affecting thymic development of murine T_{REG} cells, a T_{REG}-specific deletion of UBC9 causes an early and fatal inflammatory disorder at 3 weeks of age (136), mimicking the dynamics observed in *scurfy* mice (96). These T_{REG} cells show defects in TCR activation, migration, and peripheral accumulation (136). However, we do not observe such dramatic outcomes when knocking out *Irf4* in murine T_{REG} cells, suggesting other factors may compensate for the loss of *Irf4*. Mice harboring a conditional knock-down (*Foxp3*^{CRE} *Irf4*$^{fl/fl}$) develop spontaneous dermatitis, blepharitis, and lymphadenopathy disease by 5-6 weeks, and die by 3-4 months from a mostly T_H2-mediated autoimmune disease (133). Co-immunoprecipitation of Irf4 and Foxp3 shows that both TF interact to, facilitate the transcription of genes such as *Icos*, *Il1rl1*, *Maf* and *Ccr8* (133). In addition, Irf4 allows T_{REG} cells to exert their suppressive functions. For example, a knock-out or a disruption of Irf4 expression in murine or human T_{REG} cells, impacts the expression of key suppressive genes, including *Il10* (137). Moreover, while there is evidence Irf4 is an important contributor during the early transcriptional events involved in the specialisation of activated T_{REG} cells, this TF is also readily detected in some populations of memory T_{REG} cells in the tissue, suggesting its expression is maintained long after TCR engagement. Finally, BATF and Irf4 are particularly upregulated in relation to the strength of the TCR signal (138, 139), and, together, directly suppress *Foxp3* transcription in T_{REG} cells induced *in vitro* (139). Collectively, these observations imply that BATF and Irf4 hinder Foxp3 transcription during the early events that define eT_{REG} formation (Figure 2).

4 The unique properties of TR-T_{REG} cells

As discussed above, the pool of T_{REG} cells residing in tissues is highly dependent on the organ and is composed in adults of both TR-T_{REG} and emT_{REG} cells whose fate remains ill-defined. Moreover, while the establishment of a peripheral T_{REG} population in mucosal tissues happens in a relatively short amount of time after birth, this is not the case for VAT T_{REG} cells that follow a more gradual accumulation (27), complexifying our understanding of the events that govern TR-T_{REG} accumulation. Notably, fate-mapping systems (*Foxp3*^{eGFPCreERT2}*x ROSA26*$^{STOP-eYFP}$) in neonate mice reveal that T_{REG} cells seed non-lymphoid organs like the lungs and liver in the first 8 days of life, persisting for up to 12 weeks with little renewal (25). Critically, exposure to an inflammatory event prior to day 8, but not after, significantly reduces TCR diversity of liver and lung TR-T_{REG} and causes long-lasting alterations to their transcriptional program (25), revealing how critical the neonatal period is to the establishment of tissue homeostasis. Here, the establishment of TR-T_{REG} cells is heavily dependent on the acquisition of a core of transcriptional factors. Single-cell RNA-seq (19), bulk RNA-seq (17), microarray and ATAC-seq (16, 112) analysis of T_{REG} from visceral adipose tissue (VAT), lung, skin or colon reveal the epigenetic and transcriptional landscape of these cells is primarily determined by the organ, with only a small set of core genes shared between them. In various non-lymphoid tissues, TR-T_{REG} cells express a shared a set of core genes, including *Il1rl1* (ST2), *Gata3, Tnfrsf4, Rora*, *Il10* and *Gzmb* (16, 19). On the other hand, there is a significant

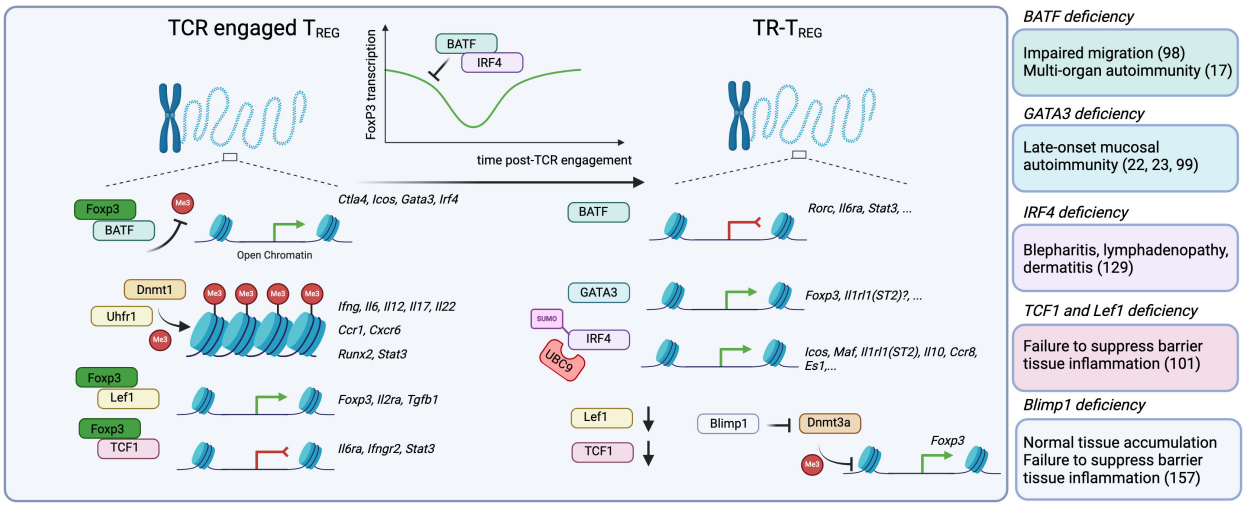

FIGURE 2
The acquisition of a tissue-resident program requires a series of epigenetic and transcriptional changes that involve modulation of Foxp3 expression or activity. After thymic egress into the periphery, T_{REG} cells are TCR-activated by self or non-self-antigens, and undergo a series of epigenetic and transcriptional changes that guide their maturation into TR-T_{REG} cells. While not entirely understood, this process seems to happen in a step-wise manner. First, TCR-engaged T_{REG} cells upregulate key transcriptional programs in part driven by the transcription factor BATF, which, in conjunction with Foxp3, promotes the accessibility of Foxp3 and expression of BATF-driven genes including *Ctla4, Icos, Gata3, Irf4*. Key to the stability of their epigenetic landscape, T_{REG} cells require Dnmt1 and its partner Uhrf1 to promote the methylation of CpG-rich regions and control the accessibility to inflammatory genes, including *Ifng, Il6, Il12, Il17a, Il22, Ccr1, Cxcr6, Runx2* and *Stat3*. Finally, Foxp3 partners with Lef1 to promote the expression of genes involved in its core program, including *Foxp3, Il2ra* and *Tgfb1*, and also with TCF1 to suppress the expression of genes associated with inflammation like *Il6ra, Ifngr2* and *Stat3*. Importantly, BATF and IRF4 can, in turn, suppress Foxp3 expression, a process that, while not fully understood, may enable the temporal accessibility of genes normally repressed by FoxP3. Once in the tissue, BATF enables the continued suppression of genes like *Rorc* (RORγT), *Il6ra* and *Stat3*. GATA3 promotes the transcription of *Foxp3*, but may be further involved in the expression of other GATA3-associated genes, like *Il1rl1*(ST2). IRF4 is also required for the expression of core TR-T_{REG} genes, including *Icos, Il1rl1* and *Il10*. Moreover, there is evidence that *Lef1* and *Tcf7* (TCF1) mRNA expression are significantly decreased in TR-T_{REG} cells, suggesting they are no longer required. Finally, BLIMP-1 expression is increased, and can actively inhibit the action of Dnmt3a, promoting the accessibility of key genes in T_{REG} cells such as *Foxp3*. Consistently, murine models with Foxp3-conditional deletion of BATF, GATA3, IRF4, TCF1 and BLIMP-1 reveal how critical these regulators are for the function of TR-T_{REG} cells.

difference in gene expression between the transcriptional signature and DNA methylation profile of colonic and skin-isolated T_{REG}, including increased *Dgat2*, a gene involved in lipid synthesis (16, 19), in skin TR-T_{REG} cells, revealing these cells acquire tissue-specific abilities that allow them to persist in these microenvironments.

4.1 Tissue-specific migratory properties of TR-T_{REG}

Following TCR engagement and clonal expansion, the development of TR-T_{REG} involves the adoption of migratory properties through the acquisition and loss of chemokine receptors and other adhesion molecules. Indeed, as they undergo deep transcriptional changes and rapid clonal expansion, they also begin to express chemokine receptors that lead them to egress from the lymph node and migrate to a selected tissue. As with other T cells, activated T_{REG} cells downregulate the surface expression of the L-selectin CD62L and upregulate the expression of the glycoprotein type I CD44 (132). Similarly, T_{REG} cells from human tumors (140) and skin (141), as well as murine T_{REG} cells isolated from multiple non-lymphoid organs (15), display low levels of CCR7, preventing their recirculation in lymphoid organs (142). However, the combination of chemokine receptors TR-T_{REG} cells possess is specific to the type of tissue these cells travelled to. In adult mice, RNA sequencing of two distinct populations of T_{REG} cells isolated from barrier tissues reveals that CCR7$^-$ T_{REG} possess an organ-specific chemokine receptor signature, regardless of their expression of the IL-33 receptor ST2 (17), suggesting that the migration of all TR-T_{REG} cells is determined by a shared group of chemokine receptors. This combination of chemokine receptors can also be appreciated in the seminal work by Miragaia and colleagues, as they observed skin-localised T_{REG} cells preferentially expressed *Ccr6*, while colonic T_{REG} cells displayed higher levels of *Ccr1* and *Ccr5*; yet, both subsets showed similar levels of *Ccr4*, *Ccr8* and *Ccxr4* (19). Unfortunately, we have yet to determine which combination of chemokine receptors is part of their migratory program and which are locally upregulated to provide further movement inside the tissues.

4.2 Core transcription factors of TR-T_{REG} cells

Interestingly, while these experiments highlight the transcriptional diversity of TR-T_{REG}, so did they help identify a core identity that govern their residency program (19). Some members of this list include transcriptional regulators that have been clearly associated to tissue

residency in other T cell subsets, like tissue-resident T_{RM} $CD8^+$ cells (143, 144), including *Runx3* and *Blimp1* (145). In addition, murine and human TR-T_{REG} also possess unique key markers including transcription factors *Ikzf2, Gata3*, and *Rora*.

4.2.1 Helios

An important transcription factor associated with TR-T_{REG} cells is Helios. While the majority of T_{REG} cells in circulation readily express Helios, siRNA-mediated silencing of Helios expression in human and murine T_{REG} cells does not impede their survival and suppressive capacity *in vitro* (146, 147). On the other hand, the conditional deletion of Helios in murine T_{REG} cells (*Foxp3*^{CRE} *Ikzf2*$^{fl/fl}$) leads to the development of a progressive, rather than a *scurfy*-like, lymphoproliferative disease in adult mice (147), revealing it is not required for the development of T_{REG} cells, but rather for the preservation of T_{REG} cell fitness at barrier tissues. Importantly, Helios potentiates the suppressive function of T_{REG} by directly interacting with Foxp3 and promoting histone deacetylation (148), providing further evidence Helios plays a supportive role to the program provided by Foxp3.

However, not all lymphoid and tissue-resident T_{REG} cells express Helios. Originally thought to be solely expressed by tT_{REG} cells (30), it is now well-appreciated that Helios expression in both murine and human Helios$^-$ T_{REG} cells is inducible (31, 149) *in vivo* and *in vitro*, respectively. Some of the key features that differentiate splenic Helios$^+$ from Helios$^-$ T_{REG} is the little overlap they share between their respective TCR repertoire, and the expression of genes involved in the differentiation of specialized T_H17 cells, including *Rorc*, *Il6ra* and *Il23r* (31), suggesting a division of labor between two T_{REG} subsets that may have long-reaching consequences in the tissue adaptation of TR-T_{REG} cells. For example, Cruz-Morales et al. showed that colonic Helios$^+$Gata3$^+$ T_{REG} differ greatly from Helios$^-$ RORγT$^+$ T_{REG} cells by their requirement of CD28, but not MHC-II, to proliferate locally (20), providing a potential point of distinction between colonic Helios$^+$ TR-T_{REG} and RORγT$^+$ emT_{REG}. Nonetheless, further investigation into the role of Helios in the differentiation and maintenance of TR-T_{REG} cells is required.

4.2.2 Gata3

Gata3 is the transcription factor 3 of the Gata-binding family that comprises six known members. In T cells, it has been shown to govern T cell development, proliferation and maintenance (150) and is particularly important to promote the transcriptional signature of helper type 2 T cells (T_H2) (151). Skin, gastro-intestinal, visceral adipose tissue, and pulmonary TR-T_{REG} cell were all shown to express Gata3 (22, 152), albeit with different intensities. This observation could be explained by the different states of activity of these T_{REG} cells, as Gata3 expression is significantly increased in both murine and human T_{REG} cells upon TCR engagement (22). Interestingly, the signaling pathway that leads T_{REG} to express this TF does not require IL-4 – a cytokine associated with Gata3 expression in conventional T cells (153) – and depends largely on exogenous IL-2 (22). Deletion of Gata3 in murine T_{REG} cells does not lead to the development of spontaneous autoimmunity before 6 months of age (22), after which the mice develop intestinal pathology and dermatitis (104). This is because Gata3-deprived TR-T_{REG} are not hindered in their development, but rather fail to respond to an inflammatory threat, displaying decreased tissue migration, proliferation, transcriptional stability, and suppressive capacity (22, 23, 104).

While not necessary for the maintenance of peripheral tolerance, Gata3 contributes to the functional adaptation of TR-T_{REG} cells. Gata3 recognizes the CNS2 region of *Foxp3* (23), promoting Foxp3 activity and stabilising the transcriptional program of T_{REG} cells to avoid their conversion to pro-inflammatory T cells under stress (22). In addition, Gata3 partners with Foxp3 to form a complex that contributes to the regulation of a wide array of T_{REG}-associated genes (104). Gastro-intestinal, skin, pulmonary, and VAT TR-T_{REG} cells express the IL-33 receptor ST2 (17, 24, 154), a known target of Gata3 in T cells (155). Unfortunately, while Gata3 is known to remodel the *Il10* locus in $CD4^+$ T cells (156), the link between Gata3 and IL-10 has yet to be established in TR-T_{REG} cells. As such, there are many indicators that Gata3 is an important contributor to the tissue adaptation of T_{REG} cells, and future investigation into the epigenetic, transcriptional, and post-transcriptional impact of this TF is warranted.

4.2.3 RORα

Another gene that is consistently found in RNA-seq data from TR-T_{REG} cells is *Rora*. This gene codes for the retinoic acid receptor-related orphan receptor alpha (RORα), a transcription factor which has been found to be expressed in differentiated T cells, including T_H1, T_H2 and T_H17 (157) cells. Unfortunately, we know very little on the role of RORα in TR-T_{REG}. In T cells, *Rora* is expressed upon TCR activation, and is closely associated with the expression of their lineage defining T_H1, T_H2 or T_H17 signature (158). Similarly, RORα plays a supporting role in the transcriptional signature of TR-T_{REG} cells. For example, a Foxp3 conditional deletion of RORα does not alter the accumulation of skin localised TR-T_{REG} cells but enables the evasion of immune responses during skin treatment with MC903, a chemical inducer of atopic dermatitis (18). Thus, as with Gata3, RORα is not required during the transcriptional transformation of tissue-migrating eT_{REG} cells, but rather for their function once in the tissue.

4.2.4 Blimp 1

The B lymphocyte-induced maturation protein-1 (Blimp 1) is a transcriptional regulator that is particularly expressed by T_{REG} cells located in secondary lymphoid organs or non-lymphoid tissues (159). A conditional knock out of *Prdm1* (Blimp-1) in murine T_{REG} (*Foxp3*^{Cre} *Prdm1*$^{fl/fl}$) generates an increase in the accumulation of T_{REG} cells, accompanied by small increase in T_{CONV} cell abundance that is insufficient to induce autoimmunity (159), confirming Blimp-1 is not essential to the generation, migration or even function of eT_{REG}. Rather, Blimp-1 prevents the methylation of multiple genes, including CNS2 in the *Foxp3* locus, by inhibiting the action of the methyltransferase Dmnt3a downstream of IL-6 (160). In doing so, Blimp-1 prevents the full conversion of colonic T_{REG} to

non-suppressive RORγT^{+} eT$_{REG}$ cells (161), suggesting that the role of Blimp-1 is to preserve the transcriptional program of TR-T$_{REG}$ cells.

4.3 Tissue-specific survival mechanisms of TR-T$_{REG}$ cells

TR-T$_{REG}$ cells have shown a remarkable capacity to communicate with their immediate environment, adopting cytokine receptors, sensing molecular changes in its environment, and providing direct cell-to-cell contact with immune and non-immune cells (162). TR-T$_{REG}$ achieve this by adopting unique phenotypic characteristics, such as the ability to sense local danger signals and compete in microenvironments with limited IL-2 availability, allowing them to maintain their identity in non-lymphoid organs.

4.3.1 IL-33

IL-33 is a cytokine of the IL-1 family of alarmins constitutively expressed by endothelial and epithelial cells (163) and by activated macrophages and dendritic cells (164). The IL-33 receptor ST2 is transcriptionally upregulated and detected on the surface of TR-T$_{REG}$ (17, 120). This is consistent with the fact that the expression of *Il1rl1* (ST2) is closely associated to the expression of BATF and is part of the transcriptional signature elicited after DNA methylation in TR-T$_{REG}$ cells (16, 120). However, not all tissue isolated T$_{REG}$ express ST2 in mice at the steady state, nor do skin, lung, gut, or VAT-isolated T$_{REG}$ cells express ST2 with the same intensity (17). As such, while suggested as a marker of TR-T$_{REG}$ cells (17), there is currently no clear evidence that ST2 expression is exclusive to TR-T$_{REG}$ cells, and further investigation into this receptor is warranted. Moreover, the importance of ST2 in the differentiation and function of TR-T$_{REG}$ cells remains ill-defined. For example, while IL-33 can directly promote the homeostatic expansion of T$_{REG}$ cells (24, 165), a Foxp3-specific conditional knock-down of ST2 (*Foxp3*^{CRE}*Il1rl1*$^{fl/fl}$) does not impair T$_{REG}$ accumulation in the lungs (166). Rather, IL-33 orchestrates T$_{REG}$-mediated suppression of local γδ T (166), T$_H$1, and T$_H$17 cells during tissue injury (24, 167). To complicate things, it is unclear if these mechanisms depend entirely on the expression of ST2 by T$_{REG}$ cells (168). Indeed, innate immune cells can readily respond to IL-33 and provide proliferative signals to promote TR-T$_{REG}$ expansion and survival (169). As such, rather than providing a survival signal, ST2 may act as a sensing mechanism for local TR-T$_{REG}$ to rapidly reactivate and produce suppressing cytokines.

4.3.2 Icos

While not exclusive to TR-T$_{REG}$, the inducible co-stimulator Icos plays a crucial role in both TR-T$_{REG}$ and emT$_{REG}$ cells to maintain their identity and survival within non-lymphoid organs (21). In mice, a Foxp3 conditional knock out of Icos (*Foxp3*$^{YFP\text{-}CRE}$ *Icos*$^{fl/fl}$) does not generate autoimmunity, but rather prevents tissue-localised T$_{REG}$ cells from suppressing oxalone-induced dermatitis (170), suggesting Icos is particularly required for T$_{REG}$ cells to control tissue injury. Specifically, Icos coordinates with mTORC1 signaling to support T$_{REG}$ proliferation and the expression of suppressive molecules (171), and is particularly critical for TR-T$_{REG}$ and emT$_{REG}$ cells to persist in the absence of IL-2 signaling by providing anti-apoptotic signals (15). Together, Icos and CD28 act as potent activators of the PI3K/Akt signaling pathway that triggers the phosphorylation of the transcription factor Foxo1 (171, 172). In turn, this sequesters Foxo1 in the cytoplasm and leads to down-regulation of genes like *Klf2* and *Ccr7* (173). In the absence of IL-2, T$_{REG}$ cells become susceptible to apoptosis, highlighting the critical role of sustained Icos-IcosL signaling in their survival as they migrate to the tissue (15). On the other hand, abrogating the PI3K-activating capacity of Icos by removing a YMFM motif in its cytoplasmic tail increases VAT TR-T$_{REG}$ accumulation and function (174), suggesting that Icos may have tissue-specific roles for T$_{REG}$ cells. Thus, while there is abundant evidence that Icos promotes the activation and survival of TR-T$_{REG}$ cells, tissue-specific differences are likely at play and must be considered when investigating TR-T$_{REG}$ cell sub-populations.

4.4 The metabolic adaptation of TR-T$_{REG}$ cells

Genes involved in fatty acid β-oxidation (FAO) can be readily detected in antigen-experienced T$_{REG}$ cells isolated from non-lymphoid tissues, including in visceral adipose tissue (VAT), the skin, the colon, and the lungs, suggesting TR-T$_{REG}$ default to FAO in non-inflamed tissues (19, 120). However, these transcriptional approaches have not formally demonstrated that TR-T$_{REG}$ cells require FAO to persist in all tissues. Most of the current evidence comes from VAT-isolated TR-T$_{REG}$, which express the peroxisome proliferator-activated receptor gamma (PPARγ), a ligand-activated transcription factor. Functionally, PPARγ provides a complex signal to engage FAO in VAT T$_{REG}$ cells (175), providing them with a competitive advantage over T$_{CONV}$ cells to survive, accumulate, and function (176). This crucial metabolic strategy enables VAT T$_{REG}$ cells to catabolize long-chain fatty acids (LCFAs) from the environment, turning to FAO to sustain their demand for energy (177, 178). While this process is shared between T$_{REG}$ and T$_{CONV}$ cells, T$_{REG}$ cells utilise fatty acids differently as they do not build endogenous fatty acids from acetyl-CoA, but rely on the acquisition of exogenous fatty acids to meet their metabolic needs (179). Concomitantly, efficient lipid storage by VAT TR-T$_{REG}$ cells is essential to protect them against lipo-toxicity and to provide the metabolic precursors needed for energy generation. These include scavenger proteins such as CD36 and enzymes involved in triglyceride production, such as DGAT1 and DGAT2. Skin and VAT-isolated PPARγ^{+} T$_{REG}$ cells readily express CD36, providing them with the ability to capture and secure LCFAs (175, 180). DGAT are a family of enzymes involved in triglyceride production and lipid droplet (LD) formation that are preferentially expressed in activated T$_{REG}$ cells (181). Foxp3 itself is a strong repressor of *Glut1* (182), the glucose transporter, and favors the expression of FAO genes (178). Yet, this mechanism acts in a feed-back loop, with DGAT1 promoting Foxp3 expression by diminishing protein kinase C (PKC) activity downstream of the TCR (181, 183). Interestingly, by tracing the tissue distribution of splenic T$_{REG}$ cells with shared

TCR sequences, Li et al. demonstrated that PPARγ-expressing eT_{REG} cells localise to other non-lymphoid sites, including the skin and the liver (184), providing new translational evidence that FAO proteins are expressed by other TR-T_{REG} cells. Nonetheless, while these observations highlight the importance of FAO for VAT TR-T_{REG} cells to sustain their bioenergetic demands, it remains to be determined if this metabolic strategy is required to sustain other TR-T_{REG} cells.

5 The inflammatory adaptation of TR-T_{REG}

One of the most recent and exciting discoveries has been the observation that activated eT_{REG} can further specialize to adopt T_H1, T_H2, T_H17, and even T_{FH}-like features. Importantly, they can express master transcription factors that are part of a transcriptional program typically expressed by helper T cells, including T-bet (T_H1), RORγT(T_H17), Gata3(T_H2), and BCL6 (T_{FH}). The differentiation, migration, and tissue accumulation of functionally-specialized T_{REG} cells in tissues is a dynamic process that can occur in microbiota-rich barrier sites (10) or during tissue injury (185, 186). Indeed, contrary to the core genes necessary for the generation and maintenance of TR-T_{REG} cells, the role of these "master" transcription factors is not associated with a residency program; rather, these TFs promote a set of specialized functions that allow T_{REG} cells to suppress or orchestrate local immune responses (Figure 3). For example, single-cell analysis performed at distinct times during an Influenza A infection in mice portrays how $Gata3^+$ T_{REG} cells are progressively replaced by antigen-specific T-bet^+CXCR3^+ T_{REG} cells in the course of disease, suggesting that, contrary to the permanent presence of TR-T_{REG}, T_H1-specialized T_{REG} cells are generated concurrently with the antiviral T_H1 response and follow the pattern of accumulation of these cells (185, 187).

Interestingly, some of these specialized T_{REG} cells ($ROR\gamma T^+$ T_{REG}) are present at the steady-state in mucosal tissues such as the colon, blurring attempts at defining what constitutes the *bona fide* TR-T_{REG} phenotype in these tissues. Indeed, key events leading to the generation of specialized T_{REG} cells include the requirement for TCR signaling and aerobic glycolysis to facilitate clonal expansion and differentiation (188). Moreover, Irf4 (27) is a necessary stepping-stone for the differentiation of specialized T_{REG} cells (9, 28, 29). A typical example of these specialized T_{REG} cells is observed in the colon, where resident T_{REG} cells displaying two distinct TCR repertoires can be segregated based on their transcriptional program. Indeed, both $ROR\gamma T^+$ T_{REG} and $Gata3^+$ T_{REG} are readily detected in the colon; however, absence of a local microflora only hinders the specific generation of $ROR\gamma T^+$ T_{REG} (189, 190) since their TCR repertoire is largely biased towards bacterial antigens (72, 191, 192). Since specific signals are required for T_{REG} cells to acquire these programs, it is possible to dissect the required pathways that lead T_{REG} cells to acquire these specialized programs.

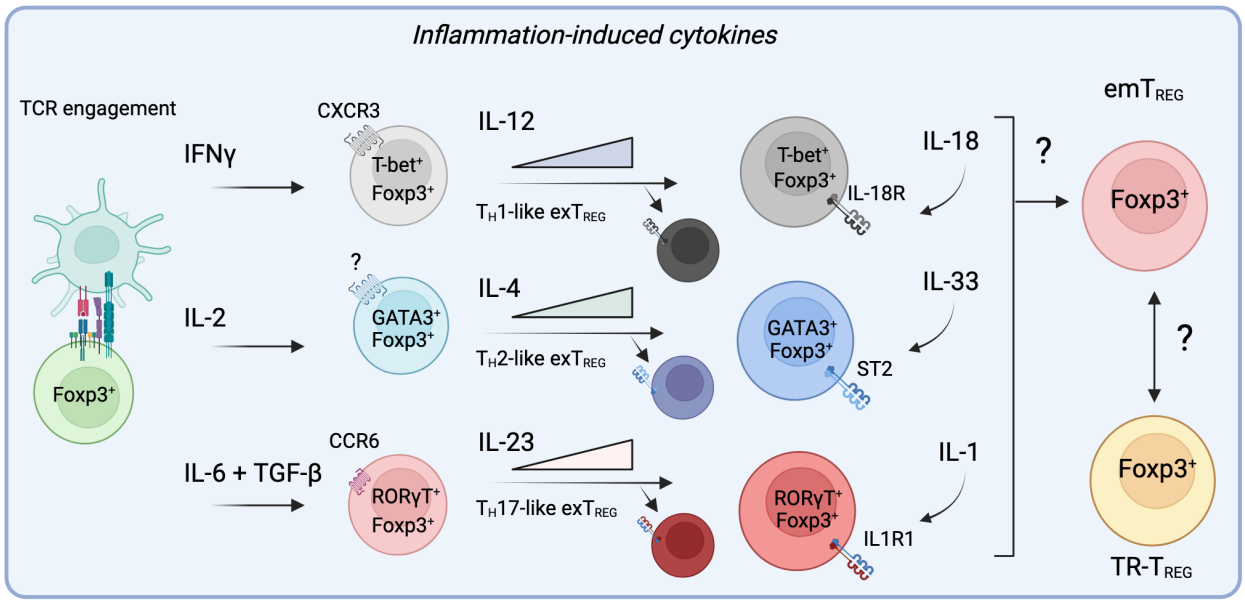

FIGURE 3
Specific inflammatory signals alter the trajectory of T_{REG} cells in non-lymphoid sites by engaging specialized programs prior and during their migration to inflamed tissues. During active inflammation, the presence of cytokines such as IFNγ, IL-2, IL-6, and TGF-β can divert the differentiation of T_{REG} cells to adopt helper-like phenotypes, allowing them to migrate to specific sites of inflammation alongside conventional T cells. Importantly, by acquiring these master transcription factors, effector T_{REG} cells (eT_{REG}) become responsive to signals provided by IL-12, IL-4 or IL-23. While these cytokines further promote the transcriptional program engaged by these specialized T_{REG} cells, they can ultimately diminish their suppressive functions and allow them them to contribute to inflammation as exT_{REG} cells. Importantly, it remains to be determined if the resulting population of emT_{REG} cells in the tissue after inflammation acquire a residency program that lead them to form part of the TR-T_{REG} cell population.

5.1 The effects of polarizing signals on the fate of T_{REG} cells

Some of the better described signals that promote the generation of specialized T_{REG} cells include cytokines that drive the phosphorylation and nuclear translocation of STAT and SMAD proteins (193). In turn, these signals promote the expression of genes that define T cell fate, including the acquisition of master transcription factors T-bet, Gata3, or RORγT. What is particularly interesting, however, is that the pathways that lead T_{REG} to adopt these TFs can also undermine their Foxp3-dependent transcriptional program, either through the loss of Foxp3 expression, the expression of pro-inflammatory genes, or the engagement of apoptosis. As such, at the time when activated T_{REG} cells undergo important epigenetic and transcriptional changes, certain inflammatory signals can promote the loss of Foxp3 expression and their conversion into inflammatory "exT_{REG}" cells. Several key transcription factors have been described to be involved in this inflammatory adaptation process of T_{REG} cells.

5.1.1 T-bet$^+$ eT_{REG}

T-bet is a T-box transcription factor expressed in a wide variety of immune cells, and mostly recognized for its role in defining the transcriptional landscape of T_H1 cells (194). Using a unique murine model that enables the tracking of murine T-bet-expressing T_{REG} (*Foxp3*$^{Thy1.1}$ *Tbx21*$^{tdTomato\text{-}T2A\text{-}CreERT2}$ *R26YFP*$^{-fl\text{-}stop\text{-}fl}$), Levine and colleagues showed that the conditional deletion of T-bet in Foxp3$^+$ T_{REG} cells does not lead to autoimmunity in adult mice, although it does generate a mild increase in T_H1 activity (195), suggesting T-bet has little to no impact on the way T_{REG} preserve tissue function at the steady-state. Notably, T-bet is a critical regulator for the expression of CXCR3 (196), a chemokine receptor that orchestrates eT_{REG} migration to sites of T_H1-driven inflammation (196, 197). Highlighting the role of TCR engagement, T-bet$^+$ eT_{REG} cells that progressively accumulate in the lungs of mice infected with acute Influenza A infection recognize viral proteins (185, 198). Thus, as with T_H1 cell polarization, the generation of T-bet$^+$ eT_{REG} occurs progressively during inflammation and is closely associated to the clonal expansion of antigen-specific CD4$^+$ T_H1 cells.

The signals that promote the generation of T_H1 cells include IFNγ (STAT1) and IL-12 (STAT4). Interestingly, an IFNγ-STAT1 signal drives the initial expression of T-bet during TCR engagement, while a subsequent IL-12-STAT4 signal is required for their definitive differentiation (199, 200). This initial T-bet expression can, in turn, promote the expression of the IL-12 receptor (IL-12Rβ2) (201–203). However, contrary to T_H1 cells, eT_{REG} cells seem to depend exclusively on the presence of IFNγ for the acquisition of T-bet (196, 204). By activating murine CD4$^+$Foxp3$^+$ cells *in vitro*, Koch and colleagues demonstrated that T_{REG} cells acquire T-bet expression and its associated target, CXCR3, only if they possess the receptor IFNγR1 (205), suggesting that IFNγ-producing T_H1 cells are responsible for the polarization of T_H1-like eT_{REG} cells.

The control of IL-12 signalling by T_{REG} cells is critical, as excessive pSTAT4 can lead T_{REG} cells to lose Foxp3 expression (206) by, notably, limiting chromatin accessibility of STAT5 to the *Foxp3* locus (207). Yet, STAT4 is a major regulator of *Ifng* in CD4$^+$ T cells (208), and both human and murine T_{REG} exposed to IL-12 produce low levels of IFNγ (187, 205, 206, 209–212), revealing excessive IL-12 can still be perceived by T_H1-like eT_{REG} cells. However, contrary to STAT1, STAT4 signaling is associated with less suppressive T_{REG} cells and can even lead to the complete loss of Foxp3 expression (187, 205, 206, 209–211), suggesting T-bet$^+$ eT_{REG} are in a constant struggle to avoid the loss of genes involved in their suppressive functions. In this regard, T-bet$^+$ T_{REG} cells possess mechanisms to avoid overt STAT4 signaling. For example, IFNγ-induced T-bet$^+$ eT_{REG} cells suppress IL-12Rβ2 surface expression, preventing excessive phosphorylation of STAT4 and further T_H1-like commitment (205). Moreover, non-labelled proteomics on circulating human T_{REG} cell populations revealed that, compared to memory or naïve T_{REG}, eT_{REG} maintain low cytosolic levels of STAT4 (213).

There is growing evidence for the role of IL-18 on the function of tissue-resident T-bet$^+$ eT_{REG} cells. While the origin of IL-18R1$^+$ eT_{REG} cell remain to be fully understood, T_H1 polarizing conditions, and particularly IL-12, allow T_{REG} cells to adopt the expression of both T-bet and IL-18R1 (187), suggesting that, like for T_{conv} cells, eT_{REG} require a STAT4-dependent chromatin remodeling to express IL-18R1 (214, 215). *In vitro*, IL-18 promotes the expansion and suppressive capacity of IL-12-generated T-bet$^+$ T_{REG} cells (187), suggesting this signal can counter the destabilising effects of IL-12. *In vivo*, T-bet$^+$ eT_{REG} cells express IL-18R1 when they accumulate in the lungs during an Influenza A infection (187). Here, IL-18 enhances the production of amphiregulin in local T_{REG} cells, facilitating tissue restoration after pulmonary Influenza A infection (216). In addition, a Foxp3 conditional knock-out of *Il18r1* (*Foxp3*$^{ERT2\text{-}CRE}$ *Il18r1*$^{fl/fl}$) allowed us to demonstrate that IL-18 is specifically required for eT_{REG} cells to suppress IL-17A responses in the lungs after an Influenza A infection (187). Similarly, IL-18R1 deficiency in T_{REG} cells fails to control the onset of a T cell-mediated colitis (217) as well as inflammation in an experimental model of ovalbumin-induced asthma (218), confirming IL-18 is an important contributor to eT_{REG} function. However, these observations do not necessarily mean that IL-18R1 expression is restricted to T-bet$^+$ T_{REG}, as we have observed RORγT expression among a subset of IL-18R1$^+$ T_{REG} cells (187) and IL-18R1 expression has been described in T_H17 cells (217). Collectively, these observations illustrate how the T_H1 adaptation of eT_{REG} cells allows for the suppression of tissue inflammation.

5.1.2 Gata3$^+$ eT_{REG}

The transcription factor Gata3, which is an important component of the transcriptional program of TR-T_{REG}, is best described for its role in driving T_H2 cell differentiation (219). In both human and murine CD4$^+$ T cells, Gata3 promotes T_H2-associated genes, allowing for the expression of genes associated to their function, such as IL-4, IL-5, and IL-13 (151, 219). There are

numerous accounts of tissue-homing T_{REG} cells expressing high levels of Gata3 during acute T_H2-driven immunity, such as what is observed during asthma (220) or helminth infections (221, 222).

The signals driving GATA-3 expression in T_{REG} cells are not fully understood. Two signals have been described to be sufficient to induce Gata3 expression during T_H2 differentiation, namely an IL2/STAT5-dependent and an IL-4/STAT6-dependent signal (223–225). In homeostatic conditions, IL-2 (STAT5) is sufficient to promote the expression of Gata3 during TCR engagement (22). However, in T_H2-driven responses, T_{REG} cells require IL-4R to acquire GATA-3 expression and their T_H2-like characteristics (226). This distinction between STAT5 and STAT6-dependent induction of Gata3 may pave the way towards understanding how T_H2-like eT_{REG} cells differ from TR-T_{REG} cells. For example, mice with a Foxp3-specific conditional knock-down of *Il4ra* (*Foxp3*^{CRE} *Il4ra*$^{fl/fl}$) fail to prevent exacerbated asthma-like symptoms when challenged with house-dust-mite (HDM) (226) and helminth-driven inflammation, despite the presence of T_{REG} cells *in situ* (221).

While IL-4 can favor T_{REG} cell-mediated functions, sustained IL-4 can also force T_{REG} cells to lose Foxp3 expression and their suppressive capacity both *in vitro* (227) and *in vivo* (221, 222, 227). STAT6 can promote the activity of the histone deacetylase HDAC9, which decreases chromatin accessibility to the *Foxp3* locus (228). To prevent this, eT_{REG} cells require strategies to avoid excessive IL-4 signaling. First, by maintaining high levels of CD25 expression, eT_{REG} cells remain sensitive to IL-2, whose STAT5 signal competes with STAT6 activity (229). Second, tissue-localised T_{REG} cells prevent further commitment into the T_H2 lineage by producing the E3 ubiquitin ligase Itch (230, 231). Finally, murine *in vitro*-induced T_{REG} cells exposed to IL-4 express higher levels of the JAK/STAT inhibitor SOCS2 to prevent further STAT6 phosphorylation and the expression of pro-inflammatory cytokines (232). Thus, while it remains to be fully confirmed in tissue-resident T_{REG} cells, there is cumulating evidence that IL-4 is important for the commitment of Gata3$^+$ eT_{REG} cells, and responsible for their transcriptional destabilisation and conversion into T_H2-like ex-T_{REG} cells.

Finally, IL-33, which contributes to the proliferation of TR-T_{REG} cells (165), can also govern the function of Gata3$^+$ eT_{REG} cells during inflammation. In this regard, IL-33-responding activated T_{REG} cells where shown to produce high amounts of IL-10 and TGF-β (233), playing a key role in maintaining intestinal homeostasis (24). Similarly, ST2$^+$ T_{REG} cells promote the suppression of anti-tumor immune responses (234–236). However, IL-33 can also drive the production of the T_H2 associated cytokines IL-5 and IL-13 in pulmonary eT_{REG} cells (233, 237, 238) and interfere with their capacity to supress T_H2 responses (238). Thus, the role of IL-33 on Gata3$^+$ T_{REG} cells is specific to the inflammatory context and may depend on whether it targets TR-T_{REG} cells or eT_{REG} cells accompanying T_H2 responses.

5.1.3 RORγT$^+$ eT_{REG}

While complex and not entirely defined, the signaling events that lead T_{REG} cells to adopt a T_H17-like phenotype include some of the same polarizing JAK-STAT and SMAD signals that are required for the generation of T_H17 cells. Indeed, the promoter functions of both Stat3 (239) and RORγT (240) are required to establish a T_H17 cell transcriptional program (241), and T_{REG} cells have been shown to share part of this transcriptional program through the acquisition of these TFs (239). In the gut, RORγT$^+$ T_{REG} cells play an essential part in maintaining gut homeostasis and contribute to maintain local homeostasis by, notably, suppressing T_H17-driven responses (242). Transcriptionally, RORγT$^+$ T_{REG} cells from the mouse colon at steady-state express higher levels of *Il23r, Il1r1, Maf, Irf4*, and *Ikzf3* than their RORγT$^-$ counterparts (191), revealing they possess a unique landscape that encompasses some key T_H17-associated genes. Moreover, RORγT is required for IL-10 production by colonic T_{REG} cells and prevention of T cell-mediated colitis (191). Similarly, RORγT is required for T_{REG} cells to control T_H17-mediated autoimmune arthritis and EAE (192, 243), suggesting that RORγT expression allows emT_{REG} cells to target and suppress T_H17-driven responses. However, the role of RORγT and its impact on the transcriptional landscape of emT_{REG} cells remains ill-defined and is likely driven by the inflammatory microenvironments these cells are exposed to.

While many cytokines can promote the nuclear translocation of Stat3 in T_H17 cells, the simultaneous signals provided by TGF-β (SMAD2/3) and IL-6 (Stat3) are sufficient, *in vitro*, to induce RORγT expression in T_{REG} cells (162, 192). Interestingly, a delicate balance is achieved between the signal provided by TGF-β and IL-6. For example, TGF-β and IL-6 synergistically promote the proteasome-dependent degradation pathway of Foxp3 (244), contributing to a partial loss of Foxp3 function. Interestingly, once colonic RORγT$^+$ T_{REG} cells are generated, they display a significantly stable phenotype, with maintained demethylation of T_{REG}-specific genes like *Foxp3, Ikzf2, Ctla4, Gitr* and *Ikzf4* (Eos) (191). In fact, these cells possess intrinsic mechanisms to avoid their full conversion towards T_H17 cells. As with IL-12 and T_H1 cells, subsequent signals provided by IL-23(Stat3) can further destabilise the transcriptional program of RORγT$^+$ T_{REG} cells and even engage an apoptotic cascade in these cells (245). Indeed, *Il23r* is amongst the genes upregulated by Stat3 and RORγT (246), making RORγT$^+$ eT_{REG} particularly sensitive to IL-23 (245). In a recent report, Jacobse and colleagues demonstrated IL-23R expression is restricted to RORγT$^+$ T_{REG} under homeostatic conditions in the colon, and colonic T_{REG} cells maintain a competitive advantage over WT T_{REG} cells to survive in these conditions (245). Concomitantly, the authors demonstrate that T_{REG} cells isolated from the lamina propria of patients with active IBD express high levels of *Il23r* and pro-apoptotic genes (126), suggesting an evolutionary conserved mechanism that orchestrates RORγT$^+$ eT_{REG} survival and function.

In addition to IL-23, IL-1β was found to promote the differentiation of human CD4$^+$CD25^{high}CD127^{low} Foxp3$^+$ T_{REG} cells into IL-17-producing cells (247, 248), suggesting IL-1 may promote a pro-inflammatory phenotype in T_{REG} cells. However, the role of IL-1 in RORγT$^+$ eT_{REG} cells remains ill-defined. Through a T-cell mediated colitis model in mice, we demonstrated that a knock-out of IL-1R1 in T_{REG} cells favors an accumulation of Gata3$^+$ T_{REG} cells over RORγT$^+$ T_{REG} cells in the colon, as IL-1 directly promotes RORγT$^+$ T_{REG} expansion (167). Despite this effect, a lack of IL-1 signaling in T_{REG} cells results in more abundant

accumulation in the colon compared to WT T_{REG} (167), suggesting IL-1 is a negative signal for the maintenance of colonic T_{REG} cells. Interestingly, there are specific situations where this effect is beneficial. For example, IL-1R1$^{-/-}$ mice infected with *Cryptococcus neoformans* are particularly sensitive to the infection, as they cannot mount an effective T cell response (249). In their lungs, these mice lack RORγT^{+} T_{REG} cells and have increased ST2^{+} T_{REG} cells in the lungs compared to WT mice (167), suggesting sustained immunosuppression. To counter this, activated T_{REG} cells express high levels of the decoy receptor IL-1R2, which allows them to neutralize IL-1 signalling (250–252).

6 Conclusion

In this review, we aimed to detail some of the major elements that govern the trajectory of a precursor T_{REG}P cell to a highly specialized TR-T_{REG} cell. It is particularly interesting that the trajectory of a T_{REG} cells is, in most regards, highly like that of the conventional T cell as it undergoes further polarization prior to reaching peripheral tissues. Importantly, the epigenetic malleability of T_{REG} cells is central to their ability to perform outside of the thymus, as these transformations allow them to sense tissue-derived signals that, in turn, modulate their suppressive functions. However, while we have accumulated a lot of information in recent years, much remains to be understood on how these tissue and inflammation-specific adaptations govern the function of TR-T_{REG} cells. For example, the notion that T_{REG} cells can adopt a specific differentiation path and revert to their previous state, labelled "plasticity" (253, 254), remains to be proven experimentally.

Finally, recent reviews have addressed how *Foxp3* gene editing, IL-2 therapy, and the use of T_{REG} cells as cellular therapies represent key strategies to engage human T_{REG} cells (255). However, most of our current knowledge on TR-T_{REG} cells has not been specifically exploited by T_{REG}-targeting therapeutical approaches. There is, however, some evidence these strategies may facilitate the expression of a tissue residency program. For example, the development of muteins or low-dose therapies (256) aimed at promoting IL-2 signaling in T_{REG} cells can promote the expression of genes associated with TR-T_{REG} cell function, such as *Il1rl1*(ST2), as well as migratory and other tissue resident genes (257). Thus, it is of interest to understand how T_{REG} targeting strategies can influence both the developmental trajectory and the function of tissue resident T_{REG} cells. In addition, understanding the migratory cues that enable TR-T_{REG} cells to recognize specific tissues can have long reaching therapeutical benefits. Chimeric antigen receptor (CAR) T_{REG} cells have been proposed as a new avenue to circumvent the constraints of low T_{REG} cells numbers and the unknown TCR repertoire of T_{REG} in autoimmune or graft-versus-host (GvHD) diseases (258). However, this approach is still very novel, and, in the absence of additional modifications, is expected to suffer from the same limitations of CAR-T cells (258, 259), including failing to adopt metabolic strategies to survive, preventing exhaustion, and maintaining their function in tissues. Thus, it is by establishing a solid understanding of the entire pathway leading T_{REG} cells to adapt to non-lymphoid organs that we provide the basis for the development of better T_{REG} cell-based therapies.

Author contributions

FA: Writing – original draft, Writing – review & editing. ZL: Writing – original draft, Writing – review & editing. AB: Writing – original draft, Writing – review & editing. CP: Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Supervision.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The funds for this study came from the Canadian Institutes of Health Research (CIHR) operating grants (MOP67211).

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 19 September 2024
DOI 10.3389/fimmu.2024.1393418

OPEN ACCESS

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RECEIVED 29 February 2024
ACCEPTED 02 September 2024
PUBLISHED 19 September 2024

CITATION
Wen Z, Qiu L, Ye Z, Tan X, Xu X, Lu M and Kuang G (2024) The role of Th/Treg immune cells in osteoarthritis.
Front. Immunol. 15:1393418.
doi: 10.3389/fimmu.2024.1393418

The role of Th/Treg immune cells in osteoarthritis

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Osteoarthritis (OA) is a prevalent clinical condition affecting the entire joint, characterized by its multifactorial etiology and complex pathophysiology. The onset of OA is linked to inflammatory mediators produced by the synovium, cartilage, and subchondral bone, all of which are closely tied to cartilage degradation. Consequently, OA may also be viewed as a systemic inflammatory disorder. Emerging studies have underscored the significance of T cells in the development of OA. Notably, imbalances in Th1/Th2 and Th17/Treg immune cells may play a crucial role in the pathogenesis of OA. This review aims to compile recent advancements in understanding the role of T cells and their Th/Treg subsets in OA, examines the immune alterations and contributions of Th/Treg cells to OA progression, and proposes novel directions for future research, including potential therapeutic strategies for OA.

KEYWORDS

OA, T cells, Th1/Th2, Th17/Treg, immune cells, inflammation

1 Introduction

Osteoarthritis (OA) is a common joint disease characterized by the degeneration of the articular cartilage. OA mainly involves the knee joint, hip joint, and distal interphalangeal joint. The articular cartilage, subchondral bone, ligament, joint capsule, synovium, and muscles around the joint are typically affected (1–3). Globally, OA is recognized as one of the main causes of morbidity and disability (4, 5). It has been estimated that by 2032, the proportion of people, aged 45 years and over, medically diagnosed with OA will increase from 26.6% to 29.5% (knee osteoarthritis (KOA) from 13.8% to 15.7%, and hip osteoarthritis from 5.8% to 6.9%) (6). In the early stage, OA is characterized by increased bone remodeling, a loss of bone structure, and slow subchondral bone densification (7). Chronic intra-articular inflammation and cartilage degeneration follow. Eventually, intractable joint pain and joint deformities occur, which in turn, seriously affect the quality of life and the ability to work of the patients (8, 9). Traditionally, OA was considered a non-inflammatory disease involving anatomical joint damage and reduced function caused by cartilage degeneration. The pathogenesis was mainly attributed to age, body mass, sex, and abnormal joint loading, as

well as articular cartilage damage caused by joint injury, misalignment, and other mechanical factors (10, 11). Most scholars have focused on the molecular biology of promoting cartilage interstitial synthesis, inhibiting cartilage stroma decomposition, and inhibiting chondrocyte apoptosis, as well as the biomechanics of joint injury and tissue engineering for promoting cartilage repair. To date, the potential mechanism of cartilage repair remains unclear (12–14). While researchers have acknowledged the significance of cartilage degeneration in the development of OA, there is still limited understanding of the concurrent inflammatory reaction. More in-depth research on the pathogenesis of OA is needed to advance clinical treatment. More recently, the pathophysiology of OA has shifted from a degenerative "wear" disease of the articular cartilage to being recognized as a multi-factorial disease involving all joint tissues, with an underlying complex pathophysiology (15). Although OA has historically been defined as a type of non-inflammatory arthritis, many patients with OA exhibit inflammation-related symptoms, such as morning stiffness, fever, pain, and joint effusion. Increasing numbers of studies have shown that the inflammatory mediators produced by the synovium, cartilage, and subchondral bone are associated with cartilage injury in the pathogenesis of OA (16). Therefore, OA is becoming more recognized as a systemic inflammatory disease. Moreover, OA is being described as a persistent state of low-grade inflammation, rather than a passive degenerative disease or so-called abrasive disease (17, 18).

A variety of immune cell infiltration is found in the synovium of patients with OA. This established that a relationship exists between orthopedic presentations and immunology. Consequently, the pathogenesis of OA involves immune inflammatory reactions, and thus, can be classified as bone immunology dysfunction (19, 20). The continuous intersection between immune cells and bone metabolism has attracted more and more attention to bone immunology. An understanding of the relationship between immune cells and bone metabolism is warranted. In patients with OA, the synovium often shows inflammatory cell infiltration. At present, T cell, B cell, macrophage, mast cell, and NK cell infiltration have been most commonly found in the synovium of patients with OA (21, 22). Moreover, innate immune components, such as complements, macrophages, proinflammatory cytokines, and chemokines, as well as adaptive immune cells, such as T cells and B cells, play important roles in the development of OA (15, 23, 24). Although the specific pathological mechanism of T cells in OA is not clear, the OA synovium has been shown to possess a greater abundance of T cells than a healthy synovium (20). OA has been associated with many types of T cells, including helper T cells (Th) and regulatory T cells (Treg), suggesting that abnormal Th/Treg cells may be an important factor in its pathogenesis (15). Hence, scientists are now gaining a better understanding and acknowledging the significance of immune cells like T cells in osteoarthritis. While some research has been conducted in the past, further in-depth studies on the mechanisms and pathophysiology are required to fully grasp their role. This review explored the progress of T cells and their subsets (Th/Treg) in OA, discussed the role and changes in T cells including Th/Treg in disease progression, and proposed future research directions and the potential for new OA treatment.

2 T cells and OA

T cells play an important role in maintaining body health and preventing diseases. They are the main cellular components of the adaptive immune system and are responsible for mediating cell-based immune responses to prevent the occurrence of various diseases (25). T cells originate from hematopoietic stem cells in the bone marrow and undergo differentiation, development, and maturation in the thymus, facilitated by the influence of specific thymic factors such as cytokines and hormones (Figure 1). T cells are effector cells involved in the adaptive immune response of proliferation, cytokine production, cytotoxicity, and differentiation (26). Under normal circumstances, the number of T cells and their subsets in the surrounding tissue is relatively stable. Immune abnormality is regarded as a change in the ratio or absolute value of the total number of T cells or their subsets. This immune abnormality is closely related to the occurrence and development of some diseases (27). It has been confirmed that pro-inflammatory cytokines play an important role in the pathogenesis of OA. Inflammatory responses aggravate the severity of OA by inducing cartilage degradation (28). The cell types involved in OA include osteoblasts, osteoclasts, chondrocytes, synovial fibroblasts, T cells, macrophages, and mesenchymal stem cells (MSCs) (29). Acquired immune cells, such as T cells, B cells, and NK cells, play an important role in the pathogenesis of OA (30, 31). In particular, T cells is critical in adaptive immunity. In the disease microenvironment, T cells are activated to produce a large number of cytokines and inflammatory mediators. Activated T lymphocytes are associated with the occurrence, development, and prognosis of OA (32, 33). Importantly, the immune responses associated with activated T cells or abnormal T cells are related to bone loss and bone destruction in arthritis. An increase in CD4+T, Th1, Th1/Th2 ratio, and Th17 enhance osteoclastic activities, while an increase in CD8+T cells, Treg, and CTLA-4 inhibit osteoclasts (34).Bone is a dynamic organ. It is in a dynamic equilibrium of continuous reconstruction or remodeling during the lifespan. To maintain the balance required in bone structure, osteocytes, osteoblasts, and osteoclasts coordinate and cooperate during bone remodeling (35). In arthritis, activated T cells regulate bone loss and joint destruction by regulating the equilibrium between the receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG). The expression of OPG in T cells is recruited by antigen receptors, indicating that activated T cells can affect bone metabolism through OPG and RANKL (36, 37). In a T cell-dependent mouse arthritis model, the blocking of RANKL with OPG can prevent the destruction of bone and cartilage, but cannot inhibit inflammation (38). Abnormal T cell immunity promotes the abnormal expression of inflammatory cytokines, such as TNF- α, which leads to osteoclast-mediated bone erosion and osteoporosis in autoimmune arthritis. Hence, it has become clear that the immune responses from activated or abnormal T cells induce bone loss and bone destruction in arthritis (39).

In the early stage of OA, inflammation occurs with inflammatory factor infiltration in the synovium. The degree of

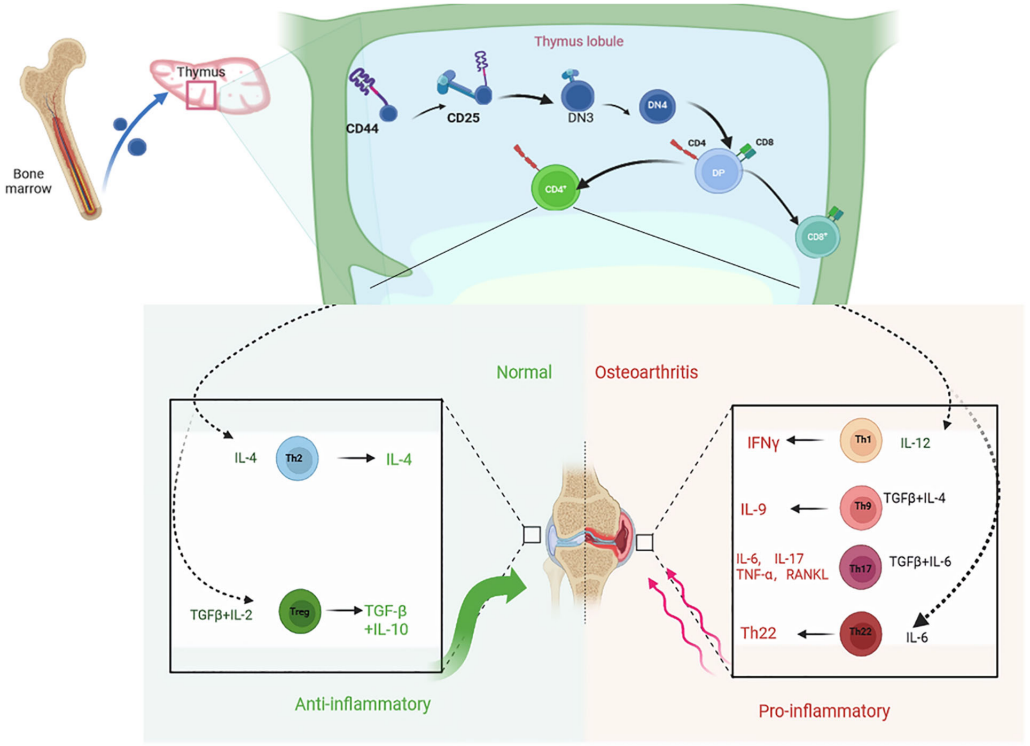

FIGURE 1
T cells originate from hematopoietic stem cells in the bone marrow and undergo differentiation, development, and maturation in the thymus. Differentiated into distinct cell subpopulations under the influence of specific thymus factors such as cytokines and hormones. Th2 and Treg cell subsets play an anti-inflammatory role by secreting cytokines IFN-γ, IL-4, IL-10 and TGF-β, delaying joint degeneration and cartilage injury, while Th1, Th9, Th17 and Th22 cell subsets promote joint inflammation.

synovitis is closely related to the symptoms of OA and the progression of the disease (40, 41). Prior research has shown that the primary immune cells found in the synovial tissue, synovial fluid, and subpatellar fat pad of individuals with osteoarthritis are T cells, macrophages, and synovial tissue resident macrophages (STRM) (42–44). In the early stage of OA, CD4+T cells induce synovitis by secreting TNF- α and IL-6, and the levels of these cytokines are significantly correlated with pain and dysfunction clinically (45). In a study using mice with gene knockout of CD8+T lymphocytes and anterior cruciate ligament transection, Hsieh et al. (46) found that the proliferation, hypertrophy, and granulation of the synovial tissue decreased on the 90th day, suggesting that there is a correlation between T lymphocytes in the synovial tissue and the progression of OA, although the specific mechanism is not clear. Scholars have found that T cell recruitment may be related to the enzymatic process. Using enzyme-linked immunosorbent assay (ELISA) to detect the supernatant of synovial cells with T cell deletion, researchers have found that T cell deletion decreases matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 levels, indicating that activated T cells in the synovium can induce the release of MMP and accelerate the process of cartilage destruction (45). These results confirm that T cells can induce OA directly or indirectly by secreting cytokines. Further study into the relationship between T cells and OA may provide new ideas for enhancing the diagnosis and treatment of OA.

3 Th cells and OA

3.1 Th1 cells

Th1 cells are a lineage of the CD4 effector T cells, which can phagocytize and clear antigens by activating macrophages and other immune cells. Th1 cells play an important role in identifying and clearing intracellular pathogens, such as viruses and bacteria (47). Th1 cells mainly secrete IFN-γ, TNF-α, and IL-2 cytokines, which can promote the further proliferation of other Th cells, leading to cellular immunity (48, 49). Initial CD4+T cells differentiate into Th1 cells under the action of IL-12 and IFN-γ. Importantly, Th1 cells play an anti-intracellular pathogen role in infections (50, 51). IL-2 and TNF-α, secreted by Th1 cells, can activate osteoclasts (52). Moreover, TNF-α can delay osteoclast apoptosis, aggravate subchondral bone destruction, localize bone remodeling after bone destruction, and subsequently, lead to the formation of new osteophytes (53).In early OA, inflammation occurs in the synovium. Rosshirt et al. (33) analyzed synovium samples from 40 patients with early OA by flow cytometry. The results showed that chemokine receptor (CXCR3/CCR5), cytokine (interferon-γ, preferentially expressed in Th1 cells), and CD161 (preferentially expressed in IL-17-producing Th17 cells) were significantly increased, indicating that the infiltration of inflammatory Th1 cells in early OA. This direct cellular interaction, combined with

humoral immunity, is involved in the pathogenesis of early OA. Timo et al (54) evaluated the pain and function of the knee joint in 47 patients with OA, who underwent knee arthroplasty. The patients' peripheral blood (PB), synovium (SM), and synovial fluid (SF) were sampled and different Th subsets were analyzed by flow cytometry. The results showed that synovial infiltration of Th subtypes (Th1, Th2, Th17) was significantly related to OA-induced dysfunction. Additionally, infiltration of CCR5+ and CCR3 + Th cells in the synovium was associated with osteoarthritic knee pain and dysfunction. Lathrati et al. (15) detected Th1 cells in the peripheral blood of patients with hyaluronic acid injection by flow cytometry. It was confirmed that the level of activated Th1 cells in the treatment group was significantly higher than that in the healthy controls. Monasterio (55) confirmed that Th1 cells are enriched in OA lesions and that these cells may activate subchondral osteoclasts through the RANKL/RANK signaling pathway to accelerate the inflammatory response. Thus, resulting in further aggravation of OA in patients. In their ACLT model rat study, Castrogiovanni (56) intervened by using physical exercise as a treatment. Results from the synovial analysis revealed that the levels of IL-4 and IL-10 in the ACLT model rats were significantly higher than those in OA model rats, while the levels of TNF- α and MMP-13 were decreased. These studies confirmed that Th1 cells are closely associated with the entire OA process, especially in the early stage of OA. The presence of Th1 cells can accelerate joint inflammatory responses, leading to cartilage matrix degradation and destroying joint homeostasis.

3.2 Th2 cells

CD4+T cells differentiate into Th2 cells under the action of cytokines, such as IL-4. Activated Th2 cells can produce cytokines including IL-4, IL-5, IL-10, and IL-13. These cytokines can promote the proliferation of Th2 cells and inhibit the proliferation of Th1 cells (41, 57). However, the researchers found that only low levels of IL-4 and IL-10 could be detected in the peripheral blood and synovial fluid of patients with OA by flow cytometry (58, 59). In a study of 18 patients with OA and 13 patients with RA, the researchers found that IL-10 transcription could be detected in the synovium of patients with OA and RA, but IL-4 and IL-5 were not detected (60). In a study that examined chemokine receptors and T cells in OA, the researchers found that compared with paired bone marrow, the cells expressing CC chemokine receptor 2 (CCR2) in the peripheral blood were significantly up-regulated and T cells CXC chemokine receptor 3 (CXCR3) were significantly down-regulated. In contrast, CCR4 was not significantly up-regulated. These observations suggest a tilt in the Th2 phenotype of patients with OA (61). Another study (62) confirmed that calcitriol can affect the differentiation of T cell subsets by inhibiting the proliferation of immature CD4+T cells to Th1 cells and promoting the maturation of Th2 cells, thus affecting the balance between osteoblasts and osteoclasts. In addition, vitamin D3 increases the production of anti-inflammatory cytokines (IL-4, IL-5, and IL-10) by Th2 cells, while inhibiting their production of pro-inflammatory cytokines (IL-2 and INF- γ). In this way, vitamin D3 regulates the immune balance of Th1/Th2 and limits the destructive effect of Th1 cells on tissue. In a clinical study (63), Javad compared the peripheral blood of 40 patients with OA treated with the natural drug, Krocina TM (containing crocin) and compared the results with the same number of patients who took a placebo. Real-time quantitative polymerase chain reaction (RT-PCR) was used to detect the expression of T-BET, GATA3, ROR- γ t, and FOXP3 as transcription factors specific to T cell subsets. The results demonstrated that after Krocina TM treatment, the expression of related genes (GATA-3 and FOXP3) increased. The result was significant for GATA-3 but not FOXP3, indicating that GATA-3 is a unique transcription factor that can differentiate T cells into Th2 subsets. Furthermore, expression of the GATA-3 gene is significantly increased in patients with osteoarthritis after crocin treatment, suggesting that crocin can affect Th2 subsets and enhance the anti-inflammatory state. Although previous studies have shown that the levels of cytokines related to Th2 cells in the synovium, synovial fluid, and peripheral blood of patients with OA are low, while the expression of IL-10 is occasionally increased, these findings did not suggest that the inflammatory response of Th2 cells is not associated with the pathogenesis of OA (64). Th1 cells produce pro-inflammatory factors, such as IL-2, IFN-γ, and TNF- α, while Th2 cells produce anti-inflammatory factors, such as IL-4 and IL-10. Th2 cells promote tissue repair by secreting IL-4 to promote the function of M2 macrophages and inhibit cell-mediated production of Th1 cells. Hence, the responses of Th1 cells and Th2 cells are considered pro-inflammatory and anti-inflammatory, respectively (65). In individuals who are in good health, there is a delicate equilibrium between Th1 and Th2 cells that helps the immune system eliminate pathogens efficiently without causing too much inflammation. However, in cases of OA, this balance is frequently disrupted, leading to an increased Th1 response and a decreased Th2 response. In sum, an imbalance in the Th1/Th2 ratio can activate osteoclasts and accelerate the inflammatory response, resulting in cartilage matrix degradation and destroying the homeostasis in cartilage (66).

3.3 Th9 cells

Th9 cells are a subgroup of effector CD4+T lymphocytes, which are differentiated from initial CD4+T cells induced by cytokines, such as IL-4 and TGF-β, and can also be induced by TGF-β alone. Activated Th9 cells are mainly characterized by the production of cytokines, such as IL-9 and IL-10 (67, 68). Th9 cells mainly accumulate in the synovial fluid and peripheral blood of patients with OA. IL-9 can maintain and increase the pro-inflammatory environment of OA, which leads to the migration and proliferation of inflammatory cells (26, 62). A study of psoriatic arthritis (PsA) and rheumatoid arthritis (RA), with OA as the control group, found that IL-9 promoted the growth and survival of locally activated T cells in an inflammatory environment. Although there was far less IL-9 in OA synovium than in PsA and RA, some infiltration was observed (69, 70). The results found that the number of Th9 cells and the level of serum IL-9 in patients with OA were significantly higher than those in healthy individuals (71). IL-9 is also an important growth factor for T cells, mast cells, and hematopoietic

stem cells, and can inhibit apoptosis. Kundu-Raychaudhuri et al (69) used Western blots to detect the signal proteins related to the survival of Th9 in the synovial fluid and peripheral blood of patients with OA. The results demonstrated a high level of IL-9 in the synovial fluid and peripheral blood and suggested that the high level of IL-9 was produced by the activation of purified CD3+T cells. It has been suggested that part or all of the IL-9 in the synovial fluid and peripheral blood of patients with OA comes from CD3+T cells. Qi et al. (72) detected the number of T cells in the peripheral blood of 25 patients with OA and 13 healthy controls by flow cytometry. The results showed that the number of Th9 cells in the peripheral blood of patients with OA was significantly higher than that of the healthy controls. The level of serum IL-9 was also higher than that of the healthy controls. Moreover, the number and level of Th9 cells were positively correlated with the osteoarthritis index score (WOMAC) of Ontario and McMaster University in patients with OA and also with their clinical symptoms and joint function. Hence, the number or level of Th9 cells has been suggested as a possible marker for judging the severity of OA. Furthermore, current studies have confirmed that Th9 cells show obvious activation and aggregation in the synovial fluid and peripheral blood of patients with OA (70, 73). Overall, IL-9 cytokines can stimulate inflammatory and autoimmune responses, promote chondrocyte apoptosis, and inhibit cartilage repair, thus aggravating OA. Research findings indicate that Th9 cells may play a significant role in the development of osteoarthritis (OA), providing insights into the impact of immune response and inflammation on OA. This suggests a potential novel treatment strategy involving the modulation of Th9 cell function to control inflammation and enhance the well-being of individuals with OA. Therefore, targeted therapy for Th9 cells may offer a potentially new treatment direction for OA.

3.4 Th17 cells

Th17 cells are a unique and important subgroup of Th cells. Their function depends on the ability of the immune system to produce and secrete key cytokines, such as IL-17, IL-21, and IL-22 (74, 75). Th17 cells differentiate from resting T cells in the microenvironment where TGF-β and IL-6 inflammatory factors coexist, and play an important role in immune responses, especially those associated with inflammatory injury relating to anti-extracellular pathogen infections, and mediating autoimmunity (76). Th17 cells also play an important role in OA. In patients with OA, the number of Th17 cells and the level of serum IL-17 are significantly higher than those in healthy controls (72). IL-17, a key factor produced by Th17 cells, can destroy homeostasis within the extracellular matrix. Notably, IL-17 is a key mediator in the pathogenesis of chronic inflammatory diseases and one of the central cytokines of arthritis. IL-17 contributes to joint inflammation by promoting the production of inflammatory cytokines and attracting additional immune cells, such as neutrophils (77). IL-17 induces inflammatory cytokines, including TNF-α, IL-1β, IL-6, and matrix metalloproteinases, that can aggravate joint destruction. IL-17 can also increase the expression of RANKL, thereby activating osteoclasts, resulting in joint bone loss (55, 78). The increased expression of Th17 cells was found in the peripheral blood of patients with OA, and the concentration of IL-17 in the serum and knee joint synovial fluid of patients with KOA was positively correlated with KOA severity (KL grade). The level of Th17 cells and their cytokines have been suggested as a potentially important index for evaluating the severity of OA (79, 80). The most direct impact of IL-17 is in cellular immune responses, along with the membrane surface antigens of chondrocytes and synovial fibroblasts. Together, they promote the infiltration and tissue destruction of many kinds of immune cells, participate in the proliferation, maturation, and chemotaxis of neutrophils, and co-stimulate the activation of T cells (81). Won et al. (82) collected peripheral blood mononuclear cells (PBMC) and SF (SFMC) from healthy individuals and patients with ankylosing spondylitis (AS). In this study, the effect of C chemokine ligand 20 (CCL20) on the migration of Th17 cells was verified by a cross-hole migration experiment. The *in vivo* effect of CCL20 inhibition was evaluated using a SKG mouse model, which is primarily a model for rheumatoid arthritis (RA), rather than OA. It was found that CCL20 could significantly reduce joint inflammation by affecting the migration of Th17 cells and inhibiting CCL20. Jung et al. (83) used the collagen-induced arthritis (CIA) model, which is a prototype RA model, rather than an OA model, to study arthritis in a mouse model by collagen. The proportion of Th17 cells in the spleen of normal and high salt diet mice was detected by flow cytometry, and the expression of IL-17 in the joint and intestinal tissues was detected by immunohistochemistry. The effect of sodium chloride on the differentiation of peripheral blood mononuclear cells into Th17 in CIA mice and the contents of sodium and IL-17 in the synovial fluid of these mice were analyzed. It was found that sodium chloride aggravated arthritis by promoting the differentiation of mouse Th17 cells in a dose-dependent manner.

Research has demonstrated that pharmacological agents, including steroids and anti-tumor necrosis factor inhibitors, can impede the differentiation of Th17 cells, consequently mitigating the symptoms associated with osteoarthritis (OA). Additionally, other biologic therapies, such as anti-IL-17 and anti-IL-23 antibodies, may also be relevant in the treatment of OA. While the majority of investigations have concentrated on the role of Th17 cells in rheumatoid arthritis (RA), current evidence indicates that targeting Th17 cells may represent a promising avenue for future therapeutic strategies in the management of OA.

3.5 Th22 cells

Th22 cells are a subgroup of cells differentiated from helper T cells under the action of IL-6, IL-1β, and TNF-α (84). Th22 cells mainly express cytokines, such as IL-22 and IL-13. These cells were named Th22 because of their ability to produce IL-22 is significantly higher than that of other Th subsets (85, 86). Many studies have confirmed the role of Th22 cells in immune and neoplastic diseases (87, 88). In one study, Th22 cells isolated from RA peripheral blood and monocytes were co-cultured with macrophage colony

stimulating factor and nuclear factor receptor activator KB ligand. The results showed that Th22 cells were more effective in inducing osteoclast formation than Th1 cells and Th17 cells (85). Interestingly, Miyazaki et al. (89) observed significant infiltration of Th22 cells in the synovium of patients with active RA, but no similar phenomenon was found in patients with OA. In another study, the researchers found that the number of Th22 cells and the level of IL-22 in the peripheral blood of patients with RA and AS were higher than those of patients with OA and healthy controls. Lejon (90) analyzed the level of T cell subsets, the related cytokines, and clinical characteristics of patients with AS versus controls from northern Sweden, and confirmed that an increased Th22 level was related to AS. Ahmad et al. (91) used CXCR3-specific antagonist NBI-74330 to block T cell-mediated signal transduction in DBA/1J mice with collagen-induced arthritis. It was found that NBI-74330 could significantly reduce the expression of IL-22 mRNA in the knee joint tissue of CIA mice. The anti-inflammatory effect of NBI-74330 may be related to a reduction in Th22 cell expression. While direct evidence linking Th22 cells to OA is currently sparse, their established roles in synovitis and bone destruction in RA suggest that similar pathways may be at play in OA. However, further research is essential to explore and confirm any such associations between Th22 cells and OA. Th22 cells are known to promote inflammation and autoimmune responses by secreting cytokines like IL-22, which may have implications for the progression of OA in settings yet to be fully understood.

4 Th1/Th2 cells imbalance and its relation with OA

Th1 and Th2 cells are two types of CD4+ T helper cells that play distinct roles in the host immune response. Th1 cells primarily release IFN-γ and tumor TNF-α, which are known for their role in enhancing cell-mediated immune response and tissue inflammation. In contrast, Th2 cells predominantly generate IL-4, IL-5, and IL-13, which are responsible for regulating humoral immune response and the anti-inflammatory process (92). The balance between Th1 and Th2 cells is typically carefully controlled by the immune system to ensure its normal function. However, this balance is disrupted in various conditions such as rheumatoid arthritis (93, 94), asthma (95, 96), inflammatory skin disorders (97), and allergies (98). While there is no direct evidence linking Th1/Th2 cell imbalance to OA, this connection can be inferred by measuring Th1 and Th2 levels in OA patients. Imbalances in Th1/Th2 cells have been implicated in the pathogenesis of osteoarthritis, contributing to inflammation and disease progression (99). This suggests an increased propensity toward inflammation. Furthermore, researchers have observed a significant increase in the levels of Th1 cells and the pro-inflammatory cytokines they produce, such as IFN-γ and TNF-α, in the synovial fluid and synovium of OA patients (100). Teng (101) conducted an extensive examination utilizing bi-directional Mendelian randomization and Bayesian co-localization techniques. Their studies revealed that the upregulation of TNF-α, which is secreted by Th1 cells, stimulated the generation of various pro-inflammatory cytokines and inflammatory mediators. This process initiated a series of inflammatory reactions, leading to joint inflammation and cartilage degradation, ultimately contributing to the progression of osteoarthritis. The cytokines produced by Th1 cells have the ability to suppress the activation of Th2 cells and their associated anti-inflammatory responses (102). Subsequent research has shown a notable reduction in both the quantity and activity of Th2 cells in individuals with OA, leading to a diminished anti-inflammatory capacity and an increased prevalence of pro-inflammatory reactions, These shift in immune response dynamics hinders effective management of joint inflammation and tissue damage in OA patients (29). In chronic bone immune disorders, such as fatty-degenerative osteonecrosis (FDOJ), over 80% of a study's 197 patients exhibited metastasis of Th2 cells. Among these, 167 subjects had an elevated Th1/Th2 ratio, suggesting that the dysregulation of Th1/Th2 cells plays a significant role in immune impairment (103).

The aforementioned research indicates a potential dysregulation of Th1/Th2 cells in both the synovial fluid and peripheral blood of individuals with OA. This imbalance is associated with the onset and progression of the condition. Given the significance of Th1/Th2 cell imbalance in OA (Figure 2), investigating the pathophysiological mechanisms of immune cells during the early stages of OA is highly valuable, as this phase presents the greatest potential for effective treatment and intervention. Rosshirt et al (33) conducted a quantitative analysis of the migration and activation of CD4+ T cells in peripheral blood (PB), synovial fluid (SF), and synovial membrane (SM) of individuals with early osteoarthritis using flow cytometry. The study revealed a significant increase in the expression of the cytokine IFN-γ in Th1 cells, while the expression of CCR3 and CCR4, primarily associated with Th2 cells, did not show a notable increase. This observation supports the notion of an altered Th1/Th2 cell balance in early knee osteoarthritis (KOA). Certain drugs, such as Sesamol (104), can regulate the balance between cellular immune responses and Th1/Th2, thereby exerting various pharmacological effects such as anti-inflammation and immune regulation. Low molecular weight polypeptide 7 (LMP7) serves as an immune proteasome subunit that influences the proliferation and specialization of T cells and modulates the balance between Th1/Th2 and Th17/Treg subsets (105). Therefore, the dysregulation of Th1/Th2 cell balance is a significant factor in the pathogenesis and progression of OA. Modulating the equilibrium of Th1/Th2 cells represents a promising therapeutic strategy for managing OA. By conducting thorough research on the functionality and interaction mechanisms of Th1 and Th2 cells, as well as investigating novel approaches to modulate this cellular equilibrium, a fresh outlook and efficient intervention for OA treatment could potentially be established. Some scholars (106) have constructed an autoantigen type II collagen peptide (CII250-270C) and the immunomodulator leflunomide (LEF) within a phosphatidylserine liposome vaccine (CII250-LEF-PSL) as a therapeutic approach for RA. This vaccine aims to promote the activation of regulatory T cells (Treg) by inducing tolerant dendritic cells (TolDC). They found that CII250-LEF-PSL effectively stimulates the differentiation of Th1 cells, modulates the Th1/Th2

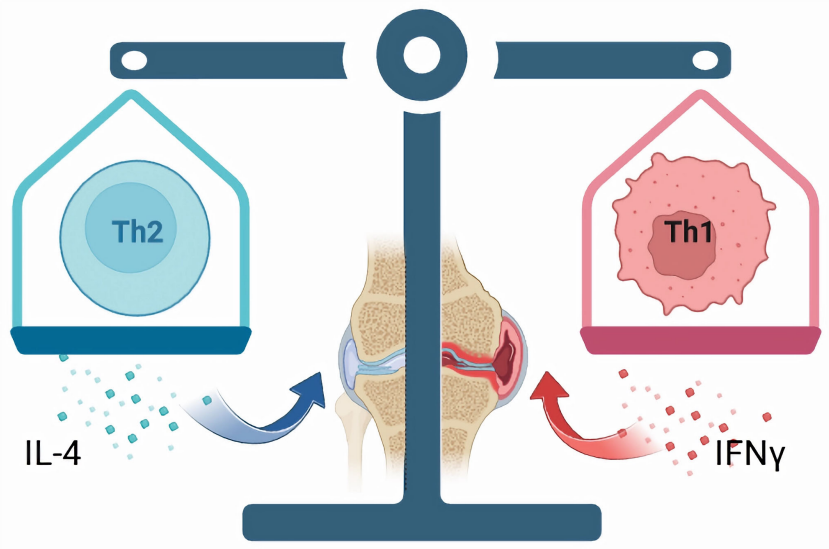

FIGURE 2
In individuals with OA, there could be a disparity in the ratio of Th1/Th2 cells, which is associated with the onset and progression of the condition. Th1 cells predominantly release IFN-γ, contributing to cell-mediated immune reactions and tissue inflammation, thereby facilitating the advancement of OA. Conversely, Th2 cells primarily generate interleukin-4 (IL-4), which plays a role in modulating humoral immune responses and anti-inflammatory mechanisms, consequently alleviating symptoms associated with OA.

balance, ameliorates synovial and cartilage damage, and consequently alleviates the symptoms of RA. Thus, the potential application of a co-delivery system involving autoantigen peptide and immunomodulator for the prevention and treatment of OA, aiming to ameliorate OA symptoms through modulation of the Th1/Th2 balance, is a promising area for further investigation.

5 Treg cells and OA

In the mid-1990s, a group of Th cells with regulatory functions was identified and named regulatory T cells (Treg) (107). Treg cells are actively controlled immune tolerance cells in the body's immune system. They play a negative role in the activation and proliferation of T cells. They contribute to the maintenance of immune tolerance, prevention of autoimmune diseases, anti-graft rejection, and tumor immunity (108, 109). Treg cells can be divided into natural regulatory T cells (nTregs) and induced adaptive regulatory T cells (aTregs or iTregs). These cells can function by interacting through direct contact with cell surface molecules rather than cytokines. For instance, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) interacts with CD80/CD86, and glucocorticoid-induced tumor necrosis factor receptor (GITR) interacts with GITR ligand (GITRL), facilitating immune regulation. Tregs can also inhibit autoimmune diseases by producing inhibitory cytokines (such as TGF- β, IL-10, IL-35) (86, 110, 111). For example, by secreting IL-10, Treg cells inhibit inflammation and autoimmune reactions, thereby contributing to immunosuppression and alleviating OA symptoms. Tregs may be an ideal cell type for the targeted treatment of OA. Kim (112) studied the use of lipid nanoparticles to regulate Treg cells in an antigen-specific manner. It was found that lipid nanoparticles can regulate the expression of cytokines and reduce the infiltration of immune cells in joints, thus inhibiting apoptosis and matrix degradation in OA chondrocytes, and relieving pain. The differentiation of Treg cells inhibited the pathogenesis of OA. In an OA rat model study, Kwon et al. (113) treated the OA rats with an intraarticular injection of Gukangning. The gene expression was detected by real-time fluorescence quantitative polymerase chain reaction and the protein expression was detected by immunohistochemistry. The results demonstrated that Gukangning inhibited cartilage osteoclasts and activated joint Treg cells, thus reducing OA pain and improving cartilage destruction. Synovitis interacts with Treg cells in the early stage of OA. Keller (114) used three horse-cultured OA models to co-culture synovial cells and chondrocytes in the Transwell system to establish normal joint and osteoarthritis models. Keller found that Treg cells can increase the expression of IL-10 and IL-4 in synovial cells and chondrocytes and increase the expression of the TIMP1 gene in synovial cells and chondrocytes, indicating their potential role in protecting cartilage. Additionally, although *in vitro* results suggest enhanced Treg function upon IL-6 blockade, further studies are needed to confirm these effects *in vivo* and assess their impact on the progression of OA. An MR study using UK Biobank and GWAS data shows that CD25, especially CD4+ and CD25+T cells, have a protective effect on OA of the hip joint (115). Clinical studies (116) have shown that in patients with osteoarthritis (OA), the frequency of CD4+CD25+Foxp3hi Tregs is significantly increased in the peripheral blood compared to healthy controls. However, the secretion of IL-10, which is also produced by Treg cells, is decreased in these patients. Importantly, this reduction in IL-10 secretion is associated with decreased expression of Tim-3 on Tregs. While both Tim-3(-) and Tim-3(+) Tregs can produce IL-10, the majority of IL-10 secretion is observed in the Tim-3(+) Treg subset. In another clinical study, the researchers analyzed Treg cell infiltration in peripheral blood (PB), synovial fluid

(SF), and synovial membrane (SM) of 47 patients undergoing knee arthroplasty by flow cytometry. Knee joint pain and joint function were evaluated and correlated with the proportion of Treg cells from different sources (peripheral blood, synovial fluid, synovium). It was found that the proportion of Treg cells in the joint samples was significantly higher than that in the peripheral blood samples. A significant correlation between infiltrating Treg cells and OA-related symptoms was also observed (117). The above studies confirmed that an imbalance of Treg cellular immunity occurs in patients with OA. Treg cells participate in the pathogenesis of OA by modulating inflammatory responses that contribute to joint degeneration. Consequently, Treg cells impact OA in significant ways.

6 The imbalance of Th17/Treg cells is an important mechanism of OA

Th17 cells and Treg cells develop from the same immature CD4+T lymphocytes. A complex relationship exists between them. Th17 cells promote inflammatory responses and represent the pro-inflammatory subsets, while Treg cells inhibit inflammatory responses and antagonize the function of Th17 cells (118). They also inhibit each other in differentiation. Studies have shown that Treg cells can inhibit the differentiation of Th17 cells by up-regulating the specific transcription factor Foxp3 or down-regulating the expression of IL-23 and IL-17. Similarly, inhibition of Th17 cells can promote the development of Treg cells (119, 120). The differentiation of CD4+T cells is a highly complex process. Activation of the cellular microenvironment and signal pathway directly determines the differentiation of CD4+T cells into the different subsets, which in turn, affects the balance of Th17/Treg cells. Th17 cells mainly secrete IL-17, which is one of the early initiating factors of joint inflammation, with a strong pro-inflammatory effect. Treg cells mainly secrete TGF-β and IL-10 to inhibit the function of self-reactive lymphocytes, thus exerting an immunosuppressive role (121–123). The balance of Th17/Treg cells maintains the balance of human immunity, and it is strictly regulated under healthy conditions (124). In the early stage of OA, the homeostasis of the joint becomes gradually out of balance under the stimulation of persistent inflammatory factors. An imbalance of Th17/Treg cells is found in patients with OA, and the proportion is closely related to OA progression (118, 125). Mansoori et al. (126) confirmed that in the ovariectomized mouse model, macrophages and CD4+T cells not only induce periodontal disease in mice but also secrete pro-inflammatory cytokines to induce NLRP3 inflammatory bodies in osteoblasts and increasing the Th17/Treg ratio, thus aggravating the formation of osteoclasts and aggravating the destruction of subchondral bone. Other researchers have confirmed that osteocytes can produce immunomodulatory cytokines through NLRP3 inflammatory bodies, change the ratio of Th17/Treg cells and osteoclast production, and thereby, aggravate the immune response, leading to bone destruction and joint degeneration (122). Ponchel et al. (71) analyzed the blood of 114 patients with OA and 121 healthy controls. In this study, Treg cells were significantly lower in patients with OA than that in the controls. The results also demonstrated that CD+T cells differentiated into Th17 cells in the synovium of those with OA. In another clinical study comparing patients with OA treated with saffron and a blank control group, the level of Th17 cells in the peripheral blood of those with OA decreased significantly, but the level of Treg cells increased (127). These studies confirmed that under normal conditions, the effects of Treg cells and Th17 cells are in a relatively balanced state, while an imbalance in the proportion of Th17/Treg cells is observed in patients with OA. IL-6 plays an important role in determining the direction of T cell differentiation. Its absence promotes the differentiation of immature CD4+T cells into Treg cells, while its existence promotes the differentiation of Th17 cells (128, 129). In sum, Th17/Treg cells have a key role in the development of OA, and the imbalance of Th17/Treg cells is involved in the pathophysiological processes of OA (Figure 3).

7 Regulating the imbalance of Th17/Treg cells is the key target for the treatment of OA

Th17 cells are pro-inflammatory CD4+ effector T cells, while Treg cells are specialized T cell with immunosuppressive and anti-inflammatory effects (130, 131). In the pathological process of OA, the balance between Th17 cells and Treg cells is disrupted, leading to inflammatory reactions and the destruction of the articular cartilage. Considering this, the Th17/Treg balance may be a potential target for new OA treatment (129, 132, 133). For example, some studies have confirmed that the proportion of Th17 cells in the synovial fluid and peripheral blood of patients with OA increases, while the proportion of Treg cells decreases, indicating that Th17/Treg imbalance plays an important role in the pathogenesis of OA. By regulating the balance of Th17/Treg cells, inflammatory responses are reduced, thus potentially relieving pain and improving joint function in models of OA, as suggested by studies (125, 134). At present, many treatments addresses the imbalance of Th17/Treg cells. Some non-steroidal anti-inflammatory drugs (NSAIDs) can inhibit the synthesis of prostaglandins, and thus, reduce the inflammatory response. Using an induction of spinal arthritis (SPA) mice model, Min et al. investigated vitronectin-derived bioactive polypeptide NPP-16 combined with celecoxib as treatment and found that VNP-16 combined with celecoxib prevented the progression of SPA by regulating the balance of Th17/Treg cells and inhibiting the expression of pro-inflammatory cytokines (135). Another study found that NSAIDs, such as ibuprofen and indomethacin, can alleviate pain and inflammation by modulating Th17/Treg imbalance in OA models (136). Some cytokines, such as IL-1, IL-6, and TNF- α, modulate the inflammatory responses in OA. Targeted drugs for these cytokines can regulate the balance of Th/Treg cells and reduce joint inflammatory responses in OA models, which may lead to pain relief and improvement in joint function (131, 137, 138). Additionally, some immunomodulatory drugs can regulate Th17/Treg balance and relieve the symptoms of OA. For example, statins can inhibit the differentiation of Th17 cells and promote the

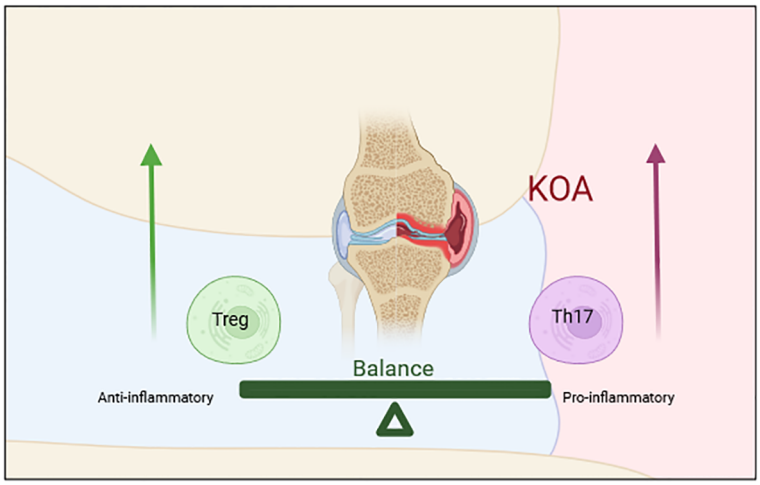

FIGURE 3
The imbalance of Th17/Treg cells is an important mechanism of OA. Th17 cells promote inflammatory responses and represent the pro-inflammatory subsets, while Treg cells inhibit inflammatory responses and antagonize the function of Th17 cells. The imbalance of Th17/Treg cells is involved in the pathophysiological processes of OA.

production of Treg cells, thus regulating Th17/Treg balance (139, 140). The differentiation of Treg cells requires the inactivation of mammalian rapamycin target (mTOR) and the activation of AMP-activated protein kinase (AMPK). Peroxisome proliferator-activated receptorγ (PPARγ) is a nuclear receptor that regulates Th17/Treg balance. Therefore, Th17 is transferred to Treg cells by activating AMPK and PPARγ, thus regulating Th17/Treg balance (129, 141). Tawfeek (142) prepared collagen-coated PCL nanofibers and characterized them by scanning electron microscope to study the effect of nanofiber scaffolds on Th17/Treg immunomodulatory properties of bone marrow mesenchymal stem cells in osteoarthritis and its mechanism. The nanofiber scaffolds enhanced the immunomodulatory effect of the bone marrow mesenchymal stem cells in osteoarthritis by increasing the expression of intercellular adhesion molecules. The treatment of Th17/Treg cell imbalance may become a key target of OA (Figure 4). It can relieve

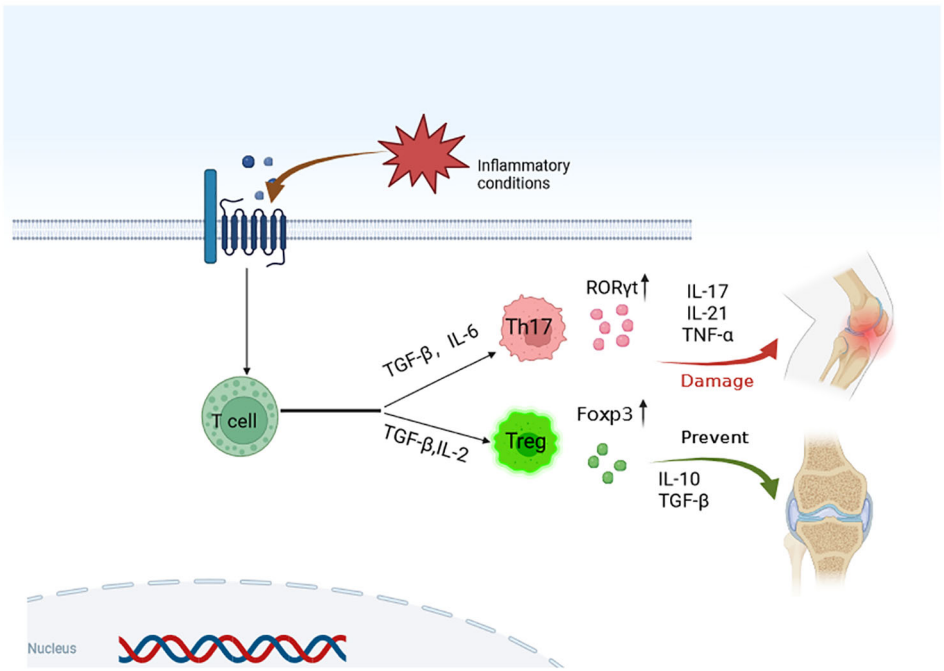

FIGURE 4
Modulating Th17/Treg cell imbalance is a key target for the treatment of OA. Through targeted regulation of cytokines and transcription regulators, the differentiation of T cells is regulated, and the activation and function of Th17/Treg cells are affected, so as to achieve the purpose of OA treatment.

pain and improve joint function by regulating Th17/Treg balance and reducing inflammatory response in OA patients. Therefore, regulating the imbalance of Th17/Treg cells is the key target for the treatment of OA. Intervention at different levels that target different cytokines, transcriptional regulatory factors, and apparent modifications can affect the activation and function of Th17/Treg cells. By regulating the inflammatory environment, OA symptoms are improved. These targets should be considered as potential new targets for the treatment of OA.

8 Discussion and prospects

Th and Treg cells are important subsets of T lymphocytes. They are important in the pathogenesis of OA. Th cells mainly include Th1, Th2, Th9, Th17, and Th22 subsets, which regulate immune responses by secreting different cytokines. Th1 cells are mainly involved in cellular immune responses, Th2 cells are mainly involved in humoral immune responses, and Th17 cells are mainly involved in inflammatory responses. Treg cells are important immunomodulatory cells, which can inhibit immune responses and maintain immune homeostasis. In OA, there is also a complex interaction between Th/Treg cells and other immune cells. In the presence of an imbalance inflammatory reactions and joint injury arise. For example, Th1 and Th17 cells can promote the activation and differentiation of macrophages, which in turn, promote inflammatory responses and joint injury. Conversely, Treg cells can inhibit the activation and differentiation of macrophages, thus inhibiting inflammatory reactions and joint injury. Th/Treg cells can also interact with other immune cells, such as B lymphocytes and natural killer cells, to regulate immune and inflammatory responses. An increased level of inflammatory factors, such as IL-1, IL-6, and TNF- α in the synovial fluid of patients with OA can activate and increase Th1 and Th17 cell functions, resulting in inflammatory responses and joint injury. while Treg cells inhibit inflammatory responses and joint injury by secreting anti-inflammatory factors, such as IL-10. The activation and function of Th/Treg cells can be affected by regulating the joint inflammatory environment. By optimizing Th/Treg cell functions, the symptoms and pathological changes of OA may be improved. In conclusion, the dysregulation of Th1/Th2 and Th17/Treg ratios is a crucial factor in the development of OA. By delving deeper into the mechanisms of these cell populations and their interactions, we can uncover fresh insights and potential targets for the early detection and treatment of OA (143).

As Th/Treg cells play a key role in the pathogenesis of OA, targeted therapy to optimize Th1/Th2 and Th17/Treg cell balance may be a potentially new strategy for OA therapy. At present, drugs, such as anti-tumor necrosis factor (TNF) inhibitors, are being used to treat patients with OA. These drugs can inhibit inflammation and relieve symptoms, such as pain. Further studies to determine if common treatment of OA, such as glucosamine and chondroitin sulfate, can promote the repair and regeneration of chondrocytes by regulating the imbalance of Th/Treg is warranted. Other drugs that can regulate the activation and function of Th/Treg cells by inhibiting or promoting the expression level of specific molecular markers should also be considered. In the future, research is needed to explore the application prospect of Th/Treg balance therapy in the treatment of OA.

Author contributions

ZW: Investigation, Software, Writing – original draft. LQ: Formal analysis, Writing – original draft. ZY: Methodology, Software, Writing – original draft. ML: Resources, Writing – review & editing. XT: Writing – review & editing. XX: Writing – review & editing. GK: Resources, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. All the authors affirmed that the study was supported by the National Natural Science Foundation (82274543 and 82174414), Hunan Provincial Natural Science Foundation(2023JJ30471). Confiscation of other commercial grants.

Acknowledgments

The figures in the article were Created with BioRender.com.

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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