REVIEW

Follicular regulatory T cells: a novel target for immunotherapy?

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Abstract

High-affinity antibodies are produced during multiple processes in germinal centres (GCs), where follicular helper T (Tfh) cells interact closely with B cells to support B-cell survival, differentiation and proliferation. Recent studies have revealed that a specialised subset of regulatory T cells, follicular regulatory T (Tfr) cells, especially fine-tune Tfh cells and GC B cells, ultimately regulating GC reactions. Alterations in frequencies or function of Tfr cells may result in multiple autoantibody-mediated or autoantibody-associated diseases. This review discusses recent insights into the physiology and pathology of Tfr cells, with a special emphasis on their potential roles in human diseases. Discrepancies are common among studies, reflecting the limited understanding of Tfr cells. Further exploration of the mechanisms of Tfr cells in these diseases and thus targeting Tfr cells may help reinstate immune homeostasis and provide novel immunotherapy.

Keywords: follicular helper T cells, follicular regulatory T cells, germinal centre responses, human diseases, humoral immunity

INTRODUCTION

Germinal centres (GCs) are secondary lymphoid organs in which somatic hypermutation, affinity maturation and class switch recombination occur, thus producing high-affinity antibodies in humoral immunity.1 During the multistep process of GC reactions, follicular helper T (Tfh) cells interact closely with B cells to support B-cell survival, differentiation and proliferation via direct contact and soluble signal.2 It was not until 10 years ago that Tfh cells were broadly acknowledged among immunologists with B-cell leukaemia/lymphoma 6 (Bcl-6) discovered as a lineage-defining transcription factor (TF) of Tfh cells.2 Although various mechanisms have been defined, the understanding of GC reactions is still elusive.

A subset of regulatory T (Treg) cells expressing C-X-C chemokine receptor type 5 (CXCR5) was first established in human tonsils.3,4 Then, in 2011, three separate articles described their physiology in mice.5–7 CXCR5+Foxp3− cells present different transcriptional signatures compared with traditional Treg cells, making them a distinct subset of Treg cells, termed follicular regulatory T (Tfr) cells. High expression of CXCR5 directs Tfr cells to the B-cell follicle by gradients of C-X-C motif chemokine ligand 13 (CXCL13), and then, they fine-tune the GC responses. They express,
simultaneously, markers of Treg cells including Foxp3, IL-10, GITR and CTLA-4 and markers of Tfh cells including Bcl-6, PD-1 and ICOS, thus possessing dual characteristics of Tfh cells and traditional Treg cells. Considering the potential expression of autoreactive T-cell receptors (TCRs), Tfr cells are more similar to Treg cells than Tfh cells. Nonetheless, Tfr cells together with Tfh cells harbour less diverse repertoires relative to Treg cells. Recent studies have investigated this population in a wide range of diseases and have gained some progress.

This review discusses the physiology, pathology, discrepancies and challenges of Tfr cells, especially their alterations and potential roles in human diseases.

**DISTRIBUTION, DIFFERENTIATION AND DEVELOPMENT OF TFR CELLS**

Follicular regulatory T cells have been found in the spleen, lymph nodes (LNs), lymph or other lymphoid tissues such as Peyer’s patches, and also in blood. Few Tfr cells are located within the GC, whereas most of them with low levels of PD-1 are located surrounding the GC. Unlike Tfh cells, which are derived from naive CD4+ T cells, Tfr cells are mainly derived from thymic Treg cells. In addition, Tfr cells are also derived from Foxp3+ precursors in a PD-L1-dependent manner using certain adjuvants. The full differentiation of Tfr cells undergoes multistage and multifactorial processes (Figure 1). A model proposed by Fonseca et al. explains that after interaction with dendritic cells (DCs), CXCR5/Foxp3+ thymic Treg cells differentiate into early-stage Tfr (eTfr) cells and then either enter the circulation or migrate to the T-B border. After interacting with cognate B cells at the T-B border, eTfr cells become intermediate Tfr (iTfr) cells. With loss of CD25 expression in GCs, signatures of iTfr cells are further strengthened into matured Tfr (mTfr) cells, which can potently suppress Tfh cells and GC B cells. There are two perspectives on the origin of circulating Tfr (cTfr) cells: one advocates eTfr cells, and the other supports lymphoid-resident mTfr cells. cTfr cells may remigrate to follicles and GCs after reactivation.

**Signals that facilitate Tfr-cell development**

T-cell receptor and costimulatory signals through CD28 and ICOS are indispensable for Tfr-cell development. Attenuated Tfr cells appear in CD28-deficient cells, CD28-deficient mice and ICOS-deficient mice. Bcl-6 is a significant TF of Tfr and Tfh cells that prompts the expression of CXCR5. Deletion of Bcl-6 in cells or in mice results in the absence of Tfr production. Foxp3 and chromatin-modifying enzyme Ezh2 prompt suppressive capacity and the transcriptional programme of Tfr cells. Once they lose Foxp3 expression, they turn into ex-Tfr cells with an aberrant transcriptional programme and impaired suppressive capacity. Ezh2-deficient Tfr cells also exhibit reduced suppressive function by altering the Tfr-cell transcriptional programme distinct from Foxp3.

The development of Treg cells requires Ca2+ influx through Ca2+-release-activated Ca2+ channels (formed by STIM and ORAI), which mediates sustained and potent Ca2+ influx and is referred to as store-operated Ca2+ entry or SOCE. Stim1/2-deficient Treg cells eliminate Ca2+ signalling and cannot differentiate into Tfr cells, which may be because of impaired TFs such as B lymphocyte-induced maturation protein 1 (Blimp-1) and signalling pathways such as CXCR5. SOCE also regulates Tfr- and Tfh-cell differentiation via NFAT2-dependent molecules including PD-1, ICOS and CXCR5 and NFAT2-mediated TFs including IRF4, BATF and Bcl-6 expression. In addition to upregulating CXCR5, Tfr cells also require NFAT2 for migration.

The role of mammalian target of rapamycin (mTOR) signalling in T-cell development and function is intricate. It is reported that mTORC1 is a negative regulator of conventional Treg-cell differentiation, while it also plays a critical role in Treg-cell homeostasis and suppressive capacity. Nevertheless, both mTORC1 and mTORC2 are indispensable for Tfh-cell development. In contrast, Roquin suppresses the PI3K-mTOR signalling, thereby inhibiting conversion of Treg to Tfr cells. In particular, mTORC1 signalling prompts early differentiation and suppressive function of Tfr cells via p-STAT3-TCF-1-Bcl-6 axis.

It is found that TRAF3, TCF1/LEF1, Id2/Id3 and ICOS/P85α-osteopontin are essential for the development of Tfr cells. The ablation of TRAF3 reduces the generation of Tfr cells by inhibiting ICOS expression. In TCF1/LEF1-conditional knockout mice, Tfr-cell development is abolished with impaired Bcl-6 expression, and single knockout results in partial reduction of Tfr cells.
Members of the helix-loop-helix family Id2 and Id3 regulate specific transcription signatures of Tfr cells by modulating CXCR5 and Foxp3 expression. The activation of ICOS promotes interaction of p85α (the regulatory subunit of PI3K) with osteopontin and thus maintains Bcl-6 expression. miR-10a may potentially promote Tfr-cell differentiation by targeting Bcl-6 and corepressor Ncor2 simultaneously, thereby diminishing the conversion of Treg cells to Tfh cells.

**Signals that inhibit Tfr-cell development**

The balance between Blimp-1 and Bcl-6 is subtle for the development of Tfr cells. Tfr cells express Blimp-1 and Bcl-6 simultaneously, whereas Tfh cells express only Bcl-6. Blimp-1 is critical for the differentiation and suppressive function of Treg cells, while Bcl-6 is suggested to be important to maintain a Tfh-like phenotype of Tfr cells. Significantly increased Tfr cells are observed in mice with Blimp-1 specifically deleted. However, Blimp-1–deficient Tfr cells attenuate the suppression of antibody generation by B cells, suggesting that Blimp-1 regulates Tfr function. An increased frequency of Tfr cells is observed in the LNs from PD-1−/− mice, and the transfer of PD-1−/− CD4+CXCR5+ Foxp3+ cells into recipient mice results in augmented differentiation and suppressive ability of Tfr cells, which is mediated by PD-L1. Therefore, the PD-1–PD-L1 pathway can inhibit differentiation and function of Tfr cell. However, PD-1−/− Tfr cells are able to home to GCs. The addition of soluble OX40L results in increased immunoglobulin generation in a
IL-21 plays multifaceted roles in impairing the number and function of Tfr cells. The percentage of Tfr cells is significantly increased in BXD2-Il21−/− mice, and the addition of IL-21 also results in defective Tfr cell–mediated suppression of GC reactions. Altogether, high concentrations of IL-21 inhibit Tfr commitment and impair their suppressive capacity while enhancing Tfh differentiation, which is mediated by downregulating p-AKT while upregulating p-Stat3. Furthermore, IL-21 can enhance B-cell metabolism and function, thus augmenting insensitivity of B cells to Tfr cell–mediated suppression. By enhancing Glut1 levels on Tfr cells, IL-21 may also alter Tfr-cell metabolism. It is conjectured that miR-15b/16 may inhibit Tfr-cell development, as they repress the expression of Rictor and mTOR, which are essential for early differentiation and effector function of Tfr cells.

The roles of miR-17–92, miR-155, IL-2, STAT-3 and AKT remain elusive. The miR-17–92 cluster is found to promote the differentiation of Tfr cells by targeting Pten and promoting PI3K/AKT/mTOR signalling using genetic overexpression cells. In addition, miR-17–92 is validated to promote Tfh-cell differentiation, and the inhibition of Pten is implicated in their early differentiation. While an increased ratio of Tfr/Tfh cells is also found in chronic GVHD mice conditionally deficient for miR-17–92, whether the underlying mechanism is attributed to selective inhibition of Tfr cells or increased apoptosis in Tfh cells deserves more investigation. miR-155 overexpression results in the lack of Tfr cells by inhibiting the expression of CTLA-4. Conversely, it is speculated that miR-155 might promote Tfr-cell differentiation by inhibiting SOCS1. High IL-2 levels preclude Tfr-cell development by promoting Blimp-1, while dnTGF-βRII II2ra −/− mice have impaired Tfr-cell development, which may be mediated by regulating Bcl-6 and Nrp-1 expression. The activation of p-STAT3 by IL-21 counteracts Tfr–mediated inhibition of Tfh cells. However, the deletion of STAT3 in Treg cells also results in loss of Tfr cells with enhanced generation of antigen-specific IgG. Likewise, mTORC1 signalling prompts Tfr-cell development by activating STAT3. AKT is required for regulating the proliferation and survival of B cells. The transfer of Tfr cells into experimental autoimmune myasthenia gravis (EAMG) mice downregulates p-AKT and thus inactivates AKT in B cells. Paradoxically, inhibition of p-AKT by IL-21 downregulates Foxp3 expression and therefore impairs Tfr-cell commitment.

**MECHANISMS OF TFR-CELL EFFECTOR FUNCTION**

Bcl6fl/flFoxp3cre mice (Tfr cell–specific depletion) exhibit lower levels of IgG, increased levels of IgA and decreased avidity to human immunodeficiency virus (HIV)-1 antigen. In addition, higher levels of IFN-γ, IL-10 and IL-21 are produced in Tfh cells from Bcl6−/− mice. The alteration in the cytokine milieu may influence the selection of B cells, ultimately resulting in abnormal GC reactions. CTLA-4 is intended to serve as a vital mediator for Tfr cells to fully exert suppressive function. Deletion of CTLA-4 results in compromised effector function of Tfr cell with accumulating Tfr cells. As a co-inhibiting molecule, CTLA-4 may downregulate costimulatory ligands B7-1 and B7-2 on antigen-presenting cells and directly control Tfh-cell differentiation by regulating CD28 engagement.

Follicular regulatory T cells inhibit the expression of specific effector genes and central metabolic (i.e. Myc and mTOR) and anabolic (i.e. serine biosynthesis and one-carbon metabolism, and purine metabolism) pathways in GC B and Tfh cells. Interestingly, such suppression is durable and lasts even in the absence of Tfr cells. The sustained inhibition is associated with epigenetic changes in B cells and can be overcome by IL-21. Follicular regulatory T cells express both the IL-1 decoy receptor IL-1R2 and the IL-1 antagonist receptor IL-1Ra, while Tfh cells express only the IL-1R1 agonist receptor. IL-1β prompts Tfh cells to secret IL-4 and IL-21; however, Tfr cells suppress the cytokine secretion to a similar extent as recombinant IL-1Ra (Anakinra). Therefore, it has been proposed that the suppressive function is mediated by IL-1R2 or IL-1Ra on Tfr cells.

Using a new TFR-DTR mouse (Cxcr5ires-LoxP-STOP-LoxP-DTR Foxp3ires-CreYFP) strain that can selectively perturb Tfr cells, it has been found that Tfr cells potently regulate early, rather than late, GC responses and control IgE production induced by Tfh13 cells in house dust mite models. In addition, Tfr cells regulate memory B-cell responses by preventing GC formation and facilitate antibody affinity during memory.
RNA sequencing has demonstrated that Tfr cells inhibit the development of a cytotoxic-like Tfh-cell subset that highly expresses Eomes proteins and granzyme B in Tfr cell–deficient (Bcl6-flox/Foxp3-cre) and Tfr cell–amplified (Blimp1-flox/Foxp3-cre) mouse strains. Since abnormal cytotoxic Tfh cells are associated with an attenuated GC and antibody responses, Tfr cells are expected to have the potential to help the GC responses.

Considering the substantial similarities between Tfr cells and Treg cells, it is plausible to extrapolate the suppressive function of Tfr cells from Treg cells such as granzyme B and suppressive cytokines. Tfr cells may induce cell death by secreting granzyme B, as Tfr cells also express granzyme B although at a lower level than Treg cells. TGF-β is speculated to be a suppressive cytokine of Tfr cells, because TGF-β signalling in T cells inhibits Tfh-cell accumulation, activates self-reactive B cell and therefore controls autoantibody production. IL-10, however, has aroused controversies in Tfr-cell effector function. Tfr cells also secrete IL-10–like Tfr cells, and IL-10+ Tfh cells can help GC responses in mice, as specific deletion of IL-10 in Tfh cells leads to impaired humoral immunity during chronic viral infection. In addition, Tfr cells suppress IL-10 production by Tfh cells and in the coculture supernatants. Consistent with its suppressive function, IL-10 inhibition impedes GC responses and humoral immunity. Nonetheless, another study found that Tfr cell–derived IL-10 promotes B-cell differentiation and GC responses by inducing nuclear FOXO1 translocation in activated B cells, contributing to the dark zone phenotype and affinity maturation during acute viral infection.

TFR CELLS IN HUMAN DISEASES AND ANIMAL MODELS

Little is known about the physiological role and mechanisms of Tfr cells because of their relatively recent studies. Nevertheless, Tfr cells have already been associated with a variety of human diseases, predominantly autoimmune diseases (AIDs; Figure 2). Considering the relative difficulty in obtaining samples from human lymphoid tissues, circulating Tfr (cTfr) cells and circulating Tfh (cTfh) cells have been regarded as the surrogate populations to investigate GC responses in most studies. Although these studies have suggested critical roles that Tfr cells play in various disease settings, many of them emphasise the correlation between numerical alterations of Tfr cells and disease manifestations, devoid of in-depth mechanism research.

Tfr cells in autoimmune diseases

Altered frequencies and/or the suppressive capacity of cTfr cells have been elucidated in a multitude of AIDs, including systemic AIDs and organ-specific AIDs (shown in Table 1). Systemic AIDs involve rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjogren’s syndrome (SS), ankylosing spondylitis (AS), IgG4-related disease (IgG4-RD) and common variable immune deficiency (CVID). Organ-specific AIDs involve multiple sclerosis (MS), myasthenia gravis (MG), Hashimoto’s thyroiditis (HT), primary biliary cholangitis (PBC), type 1 diabetes (T1D) and ulcerative colitis (UC). It is also demonstrated that when the regulatory capacity of Tfr cells is impaired, the expansion of Tfr cells is accompanied by the development of autoimmunity in mice.

Increased and decreased frequencies of cTfr cells are found in RA patients, while no significant difference is found in early-stage RA. The frequency of cTfr cells has negative or no correlation with disease activity, and the ratio of cTfr/cTfh is correlated with disease activity. Moreover, increased cTfr cells with enhanced suppressive function and activated CD45RA Foxp3high cTfr subset have been found in patients with RA in stable remission compared with patients with active RA and healthy controls. Percentages of cTfr and cTfh cells are decreased when prescribed with methotrexate.

Intriguingly, divergences are also observed in patients with SLE. Lower frequencies of cTfr cells were observed in two studies, while they were higher in another study. Despite the total reduction in one study, active subpopulations PD-1highICOShigh Tfr cells and Ki-67+Tfr cells are increased in SLE patients. The frequency of cTfr cells is negatively correlated with serum anti-dsDNA antibody levels and with disease activity and is significantly lower in seropositive anti-dsDNA patients than in seronegative anti-dsDNA patients. In contrast, another study has found a positive correlation with clinical severity and autoantibodies IgG and IgA. The suppressive function of cTfr cells is not altered in newly diagnosed SLE patients. In addition, the
imbalance of cTfr cells can be improved after effective therapy. Reduced frequency and potentially impaired inhibitory capacity of CD4<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup>Foxp3<sup>+</sup> cTfr cells are found in spleens from BXD2 autoimmune lupus mice. In the presence of NFAT2-deficient Tfr cells, the expression of CXCR5 is downregulated, and lupus-like disease is exacerbated in chromatin-immunised mice.

Elevated cTfr levels and cTfr/cTfh ratios in SS patients have reached a consensus, especially in patients with high autoantibody levels. The expression of PD-1 on cTfr cells is increased. Activated PD-1<sup>+</sup>ICOS<sup>+</sup>cTfh cells are closely associated with SS disease activity compared with patients with non-SS/sicca syndrome, and the increased ratio of cTfr/cTfh indicates the formation of ectopic lymphoid structures within minor salivary glands, typically in patients with focal sialadenitis. Furthermore, cTfr cells do not preferentially inhibit humoral responses because of the lack of full B cell-suppressive capacity, limiting class switch recombination, and they show a naive-like phenotype. These cells are not emerged from the thymus but are produced in peripheral lymphoid organs during the GC reaction, leaving the tissue and then entering the blood. Thus, the increased frequency of the cTfr cells indicates ongoing humoral activity, and the cTfr/cTfh ratio is suggested to be considered as a strong predictor of SS diagnosis and focal sialadenitis in patients suffering sicca symptoms. Impaired cTfr cells contribute to decreased saliva flow rates and enhanced salivary gland-specific antibodies, tissue destruction and IgG deposition in a mouse experimental SS model, indicative of the development of disease.

Percentages of cTfr cells and cTfh cells are significantly higher in patients with AS. In addition, the frequency of cTfr cells is negatively associated with serum IgA in AS patients before treatment and is negatively associated with cTfh cells and the level of serum IL-21 after 1 month of standard treatment in drug responders.

IL-10<sup>+</sup>-producing cTfr cells are increased in patients with IgG4-RD. In addition, the frequency of cTfr cells is positively correlated with serum IgG4, ratio of IgG4/IgG, number of organs involved and soluble IL-2 receptor in IgG4-RD patients. The percentages of blood and tonsillar Tfr cells are increased with ageing, especially at the ages of IgG4-RD high prevalence.

The frequency of cTfr cells is decreased only in CVID patients with ≤2% of IgD<sup>-</sup>CD27<sup>+</sup> (switched memory phenotype) B cells (smB<sup>+</sup>), while the
| Disease | Authors | Molecular phenotype of Tfr cells | Main findings |
|---------|---------|-------------------------------|---------------|
| RA      | Pandya et al. | CD4^+CD25^-CXCR5^+CD127^lo | cTfr-, cTfh-, cTfr^+^, cTfr/CTh^+^, activated cTfr, function of cTfr, cTfr is negatively correlated with IgG, ACPA, RF and DAS28 |
|         | Liu et al. | CD4^+CXCR5^+Foxp3^+ | cTfr^+^, cTfh^+^, cTfr/CTh^+^, cTfr does not correlate with DAS28, RF and ACPA, cTfr/CTh is negatively correlated with DAS28 |
| Romão et al. |  | CD4^+CXCR5^+Foxp3^+ | cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with autoantibodies and SLEDAI scores |
| Niu et al. |  | CD4^+CXCR5^+Foxp3^+ | cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| Wang et al. |  | CD4^+CXCR5^+CD127^lo | cTfr^+^, cTfh^+^, cTfr/CTh^+^, cTfr is negatively correlated with serum anti-dsDNA antibody level |
| SLE     | Ma et al. | CD4^+CXCR5^+Foxp3^+ | cTfr^+^, cTfh^+^, cTfr/CTh^+^, cTfr is negatively correlated with serum anti-dsDNA antibody level |
| Xu et al. |  | CD4^+CD25^+CD127^lo-intermediate-CXCR5^+ | cTfr^+^, cTfh^+^, cTfr/CTh^+^, cTfr is negatively correlated with serum anti-dsDNA antibody level |
| Liu et al. |  | CD4^+CXCR5^+Foxp3^+ | cTfr^+^, cTfh^+^, cTfr/CTh^+^, cTfr is negatively correlated with serum anti-dsDNA antibody level |
| Jacquemin et al. |  | CD4^+CD45RA^-CXCR5^+Foxp3^+ | cTfr^+^, cTfh^+^, cTfr/CTh^+^, cTfr is negatively correlated with serum anti-dsDNA antibody level |
| SS      | Fonseca et al. | CXCR5^+Foxp3^-CD4^-/CXCR5^-CD25^-CD127^-CD4^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| Fonseca et al. |  | CXCR5^-CD25^-Foxp3^-CD4^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| Ivanchenko et al. |  | CXCR5^-Foxp3^+CD4^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| AS      | Shan et al. | Foxp3^-CXCR5^+CD4^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| IgG4-RD | Ito et al. | CD3^-CD4^-CXCR5^-PD-1^-CD25^-CD127^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| CVID    | Cunnill et al. | CD4^+CXCR5^-CD25^-CD127^lo | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| MS      | Dhaeze et al. | CD4^+CD25^-CD4^-CD25^-CD127^-CXCR5^-PD-1^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| Jones et al. |  | CD4^-CXCR5^-Foxp3^- | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |
| Puthenparambil et al. |  | CD3^-CD4^-CXCR5^-CD25^-CD127^dim | cTfr^+^, cTfh^+^, cTfr/CTh^+^, the expression of PD-1 on Tfr^+^, cTfr^+^, the suppressive function is not altered, cTfr is positively correlated with serum anti-dsDNA antibody level |

(Continued)
frequency of cTfh cells is increased in both smB+ and smB− patients. Moreover, CD4+CXCR5+CD25hiCD127lo cTfr cells exert inhibitory capacity as nonfollicular CD4+CXCR5+CD25hiCD127low cells.

In MS patients, significantly lower frequencies of cTfr cells were found in two studies,15,71 as are Tfr cells in cerebrospinal fluid.71 In addition, a lower cTfr/cTfh ratio is related to higher IgG production and circulating B-cell percentage.71 Notwithstanding, in patients with early clinical phase clinically isolated syndrome, a neurological disturbance often occurs before the development of MS, and the proportions of cTfr cells and cTfh cells are not significantly different from healthy controls.72 Specifically, proportions of proinflammatory Th17-like cTfr cells15 and cytokine-producing CD45RA−Foxp3lo non-cTfr cells72 are increased, while the proportion of suppressive fraction CD45RA−Foxp3hi resting cTfr cells72 is reduced in MS patients, which may explain the impaired suppressive function of cTfr cells. This impairment may be because of a defect in CTLA-4 signalling and that the most potent Tfr cells home to the lymph organs to inhibit the ongoing GC response.15

Decreased cTfr cells are found in MG patients compared with healthy controls.73–75 The ratio of cTfr/cTfh is positively correlated with the expression of the autoimmune regulator gene in peripheral blood73 but negatively correlated with the disease severity of MG.73,74 In addition, a significantly decreased frequency of cTfr cells and an increased frequency of cTfh cells are observed in generalised MG (GMG) patients compared with untreated ocular MG (OMG) patients,74 and the cTfr/cTfh ratio is the lowest in the GMG patients, as compared to the OMG patients, and higher in healthy controls.73 After glucocorticoid treatment, cTfr cells and cTfh cells in MG patients restore immune homeostasis.74

A significantly increased percentage of cTfr cells and the ratio of cTfr/cTfh are observed in patients with HT, and Th2-like cTfr cells are significantly upregulated, while CTLA-4 is downregulated on Tfr cells, which may contribute to the impaired humoral immune function of cTfr cells in HT.76

Table 1. Continued.

| Disease | Authors | Molecular phenotype of Tfr cells | Main findings |
|---------|---------|---------------------------------|--------------|
| MG      | Wen et al.74 | CD4+CXCR5+Foxp3+ | cTfr+, cTfh+, cTfr/cTfh is negatively correlated with the disease activity |
| Cui et al.75, Zhao et al.73 | CD4−Foxp3+CXCR5+ICOS+ | cTfr+, cTfh+, cTfr/cTfh is the lowest in GMG patients |
| HT      | Zhao et al.76 | CD4+CXCR5−CD25hi/CD127lo | cTfr+, cTfr/cTfh, expression of ICOS, PD-1 on Tfr, CTLA-4 Δ |
| PBC     | Zheng et al.77 | CD4+CXCR5+CD127hiCD25hi | cTfr+, cTfh+, ICOS+cTfr+, cTfr/cTfh is inversely correlated with disease progression, drug response and level of serum IgM |
| T1D     | Xu et al.78 | CD4+CD19 Foxp3+CXCR5+ ICOS+/CD4+CD19− Foxp3+CXCR5− PD-1+ | cTfr is positively correlated with fasting serum C-peptide levels in T1D patients |
| UC      | Wang et al.79 | Foxp3+CXCR5+CD4+ | cTfr and cTfr/cTfh are negatively correlated with disease activity |

Unless stated in the corresponding text, frequencies of Tfr cells and Tfh cells and the suppressive function of Tfr cells are compared between patients and healthy subjects; ↓, lower level compared with healthy subjects; ↑, higher level compared with healthy subjects; -, no statistically significant difference between patients and healthy subjects. ACPA, anticitrullinated protein antibodies; AIDs, autoimmune diseases; AS, ankylosing spondylitis; CRP, C-reactive protein; cTfr, circulating follicular regulatory T; CVID, common variable immune deficiency; DAS28, disease activity score in 28 joint; dsDNA, double-stranded DNA; ESR, erythrocyte sedimentation rate; GMG, generalised myasthenia gravis; HT, Hashimoto’s thyroiditis; IgG, immunoglobulin G; IgG4-RD, IgG4-related disease; MG, myasthenia gravis; MS, multiple sclerosis; NA, not available; PA-IgG, platelet antibody IgG; PBC, primary biliary cholangitis; PLT, platelet counts; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; smB, switched memory phenotype B cells; SS, Sjögren’s syndrome; T1D, type 1 diabetes; Tfr, follicular regulatory T; UC, ulcerative colitis.
Y Huang et al.

Tfr cells and immunotherapy

Decreased cTfr cells and increased cTfh cells are found in patients with PBC.77 Meanwhile, both ICOS⁺cTfr cells and CTLA-4⁺cTfh cells are increased, and the ratio of cTfr/cTfh is inversely correlated with disease progression, drug response and the level of serum IgM. The effector memory phenotype (CCR7⁺PD-1⁻) in cTfr cells and cTfh cells is significantly increased, while the central memory phenotype (CCR7⁻PD-1⁻) is decreased in PBC patients, and the frequency of effector memory cTfr cells is positively correlated with the level of serum alkaline phosphatase. Based on the expression of CXCR3 and CCR6, CD4⁺CXCR5⁺T cells are further classified into three types: Tfh1 type (CXCR3⁺CCR6⁻), Tfh2 type (CXCR3⁻CCR6⁻) and Tfh17 type (CXCR3⁻CCR6⁺). The ratio of (Tfh2/Tfh17)/Tfh1 is decreased, but the ratio of (Tfh2/Tfh17)/Tfh1 is increased in PBC patients, which may imply the ongoing humoral immune response.

Significantly decreased CD4⁺CD19⁺ Foxp3⁺CXCR5⁺ICOS⁺ and CD4⁺CD19⁺ Foxp3⁺CXCR5⁺PD-1⁻cTfr cells and the PD-1⁻cTfr/cTfh ratio are found in patients with both T1D and type 2 diabetes (T2D).78 Between these two different markers, only the frequency of CXCR5⁺PD-1⁻ cTfr cells is positively correlated with fasting serum C-peptide levels in T1D patients, which is contrary to that of T2D patients. However, in only T1D patients, the frequency of Tfr cells is correlated with levels of positive autoantibodies. In addition, cTfr cells decrease significantly after 1-year follow-up with the progress of T1D. Impaired suppressive function of cTfr cells is also observed in T1D patients. Decreased cTfr cells are further validated in nonobese diabetic mice and are associated with ongoing diabetes. Notably, an adoptive transfer of Tfr cells effectively prevents diabetes onset.

Decreased frequencies of cTfr cells are observed in UC patients.79 And the subtype IL-10⁺Foxp3⁺CXCR5⁺ cells are decreased, but cTfh cells and the subtype IL-21⁺Foxp3⁺CXCR5⁻ cells are expanded in UC.

Although the emphasis of Tfr-cell studies is mainly on AIDs, vaccine responses14,15 and other diseases including infections,82–88 cancers,89–91 allergies,92,93 chronic graft rejection/grant-versus-host disease (GVHD)94 and acute respiratory distress syndrome (ARDS)95 have also been under investigation (shown in Table 2).

Tfr cells in infections

A greater proportion of Tfr cells is found in chronically HIV⁺ spleens,87 lymphoid tissues84,85 and tonsils.84,87 The expansion of Tfr cells is mediated by HIV viral replication, IDO and TGF-β signalling, enhanced proliferation and weakened apoptosis, and regulatory DC.85 Moreover, tonsil Tfr cells exhibit increased regulatory function and dysregulated activity of Tfh cells during HIV infection.85 Tfr cells are highly permissive to R5-tropic HIV-1, probably because of the elevated expression of CCR5 and an enhanced proliferative state, and the heightened permissivity leads to persistent HIV-1 replication in vivo.86 Paradoxically, increased CD4⁺Foxp3⁺CD20⁺IgD⁺ and Foxp3⁺CD25⁺CXCR5⁺CCR7⁺CD4⁺ Tfr cells,85,96 unchanged CD4⁺CD25⁺Foxp3⁺CXCR5⁺PD1hiBcl-6⁻ Tfr cells97 and decreased CXCR5⁺CCR7⁺Foxp3⁺CD25⁺CD4⁺ Tfr cells98 are discovered in lymphoid tissues from rhesus macaques during chronic Simian immunodeficiency virus (SIV) infection, and the discrepancies are presumably attributed to distinct gating strategies. In contrast to chronic infection, Foxp3⁺CD25⁺CXCR5⁺CCR7⁺CD4⁺ Tfr cells are reduced during acute SIV infection, and the decreased ratio of Tfr/Tfh, rather than the frequency of Tfr cells, is correlated with anti-dsDNA antibody and antiphospholipid antibody levels.96 In addition, the frequency of Tfr cells is negatively correlated with the avidity of antibodies recognising SIV-gp120.98 The percentage of Tfr cells is also increased during chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection,82,83,88 which is more pronounced in patients with liver cirrhosis82 and HBsAg⁺ chronic hepatitis B (CHB).88 The percentage of IL-10⁺producing tonsillar Tfr cells is increased, and the expression of CTLA-4 on Tfr cells is upregulated.83 In addition, the frequency of cTfr cells and the ratio of cTfr/cTfh are positively correlated with fibrosis index based on four factors and the aspartate transaminase-to-platelet ratio index.82 Conversely, one study found that an increased number of cTfr cells are accompanied by higher levels of serum HBV DNA, HBsAg and serum alanine transaminase (ALT) in CHB patients and HCV RNA and ALT in chronic hepatitis C (CHC) patients,88 while another study indicated no correlations between cTfr cells and HBV DNA and ALT.82 The suppressive capacity of Tfr cells against Tfh cells is enhanced after exposure to HCV.83 The percentage of cTfr cells is also increased in patients with schistosomiasis, and most are CD45RA⁻ cTfr cells, reflecting memory-like properties.86 It seems that the increase in Tfr cells in infections has gained consensus in humans.
Increased frequencies of Tfr cells and Tfh cells are found in tumor-draining LN s and peripheral blood from patients with breast cancer, non-small-cell lung cancer (NSCLC) and ovarian carcinoma. Tumor-infiltrating Tfr cells exhibit a higher proportion than Tfh cells. In addition, Tfr cells from ovarian cancer patients exhibit higher TGF-β (TGFBI) and IL-10 (IL10) gene transcription levels, particularly the tumor-infiltrating Tfr cells. The frequency of Tfh cells is not correlated with metastasis or the progression of breast cancer, whereas the frequency of Tfh cells is correlated with clinical stage and histological subtypes of NSCLC patients. Notably, when cocultured with CD8+ T cells in ovarian cancer patients, Tfr cells inhibit the activation of CD8+ T cells in an IL-10-dependent manner, indicative of enhanced suppressive function.

### Tfr cells in allergies

Frequencies of Tfr cells are found to be lower in peripheral blood from post-transplant food allergy patients and in tonsils from allergic
Tfr cells in rhinitis (AR) patients. The phenotype and number of cTfr cells are correlated with tonsillar Tfr cells. Moreover, cTfr cells in AR patients show impaired function specifically in suppressing IgE expression rather than other immunoglobulin types. The number and function of cTfr cells are negatively correlated with antigen-specific IgE production and disease activity in AR patients. Once patients get relief after allergen immunotherapy, the frequency and function of cTfr cells are recovered. Tfr cells have also been investigated in a food allergy mouse model to measure peanut-specific antibody responses.

Tfr cells in other disease settings

In renal transplant patients with chronic renal allograft dysfunction, frequencies of Tfr cells in peripheral blood and renal grafts from those with antibody-mediated rejection (AMR) are significantly decreased, while the frequency of IL-21-producing Tfh cells is increased, relative to those non-AMR patients. However, Tfr cells exhibit equivalent suppressive function between AMR and non-AMR patients. An increase in the Tfr-cell ratio inhibits the expression of IL-21 in Tfh cells, the proliferation and differentiation of B cells, and IgG and IgA secretion of plasma cells. The miR-17–92 cluster is proved to facilitate Tfh-cell differentiation and impair Tfr/Tfh balance, thus accelerating the development of chronic GVHD in mice. In addition, Tfr cells prompt lymphangiogenesis and Breg proliferation, exerting an atheroprotective effect in Apoe mice transferred with Tfr cells.

Follicular regulatory T cells are significantly elevated in peripheral blood mononuclear cells (PBMCs) and in mini-bronchoalveolar lavage (BAL) during the onset of ARDS. Notably, Tfr cells account for a small proportion of Treg cells in PBMCs and a major proportion in mini-BAL. Compared with non-Tfr Treg cells, Tfr cells exhibit lower levels of CTLA-4 and IL-10 and weaker suppression capacity towards autologous CD4+CD25+ T cells but enhanced capacity to induce IL-10+Breg cells.

Tfr cells in ageing

The expansion of Tfr cells in ageing is corroborated in patients with NSCLC and IgG4-RD. The percentages of cTfr cells and cTfh cells are higher in patients older than 60 years, but the cTfr/cTfh ratio is decreased with age. Nevertheless, the suppressive capacity of Tfr cells decreases with ageing. Both Tfr cells and Tfh cells expand with age in mice, and Tfr cells are more pronounced. However, male mice exhibit a higher percentage of Tfr cells than their female counterparts regardless of age. Aged and young Tfr cells exhibit identical suppressive capacity but a distinct phenotype with enhanced PD-1 and decreased ICOS expression.

CHALLENGES AND UNSOLVED QUESTIONS OF TFR CELLS

Despite the fact that great progress has been made in the physiology of Tfr cells in recent years, much is unknown, and significant phenotypic and functional heterogeneity of Tfr cells still exists among various disease settings. The uniform methodology to identify and purify Tfr cells is a technical challenge. Actually, the defining markers such as Foxp3, CD25, and CXCR5 are also upregulated during effector T (Teff)-cell activation. Unlike Treg cells, recent studies have shown that bona fide Tfr cells do not express IL-2Ra (CD25), which is inconsistent with previous description of the CD25+Tfr cell. Thus, most previous studies investigating the physiology of Tfr cells may be mixtures of Tfr cells and Treg cells. Notably, both CD25+ and CD25- Tfr cells exist in follicles and GCs. Therefore, it is imperative to designate corresponding markers in different anatomic locations. Tfr cells were analysed initially by total Treg depletion, adoptive transfer along with other T cells, and then mice with deletion of Roquin and NFAT2, but these studies may manifest nonspecific effects or nonphysiological function of Tfr cells. Recently, a novel model using Bcl6fl/fl Foxp3cre mice has been under investigation, and the findings about Tfr-cell function are sharply distinct from previous studies. Nevertheless, Bcl6fl/fl Foxp3cre mice show increased Tfr-cell function in atopic dermatitis, suggesting that Tfr cells have a protective role in inflammatory diseases.
mouse model has its limits, and a more recent study has used a Cxcr5\textsuperscript{IRES-LoxP-STOP-LoxP-DTR} Foxp3\textsuperscript{IRES-CreYFP} mouse strain.\textsuperscript{52} Therefore, the establishment of uniform markers both phenotypically and functionally to unambiguously define bona fide Tfr cells in corresponding anatomic locations and of models to analyse Tfr cells specifically and physiologically will greatly deepen our understanding of Tfr cells.

Restricted by the samples, cTfr cells are often chosen as surrogate indicators, but the origin, phenotype and function between cTfr and GC Tfr cells have not yet reached a final conclusion (shown in Table 3). However, the kinetics of LN Tfr cells are found to be similar to those of cTfr cells.\textsuperscript{18} It is acknowledged that cTfr cells are derived from lymphoid tissues and are at least activated by DC.\textsuperscript{14–16} Two studies conducted in patients with X-linked agammaglobulinaemia,\textsuperscript{14} and anti-CD20 antibody rituximab treatment,\textsuperscript{107} both B cell–deficient, found that cTfr- and cTfh-cell populations are not influenced. In addition, cTfr cells comprise a phenotypically distinct population compared with GC Tfr cells, displaying similar or lower levels of CXCR5 and lower or even no ICOS, PD-1 or Bcl-6.\textsuperscript{14–16} Resembling,\textsuperscript{15} attenuated\textsuperscript{16} and incomplete\textsuperscript{14} effector function of cTfr cells is described by sorting cTfr cells with distinct strategies. RNA-seq has also validated that cTfr cells separate from GC Tfr cells, and even LN Tfr cells only partially overlap with splenic Tfr cells in mice immunised with NP-OVA.\textsuperscript{19} More specifically, LN Tfr cells exhibit higher levels of ICOS and CTLA-4, similar levels of Ki-67, but lower levels of PD-1 relative to splenic Tfr cells. When comparing their suppressive ability, LN Tfr cells are more potent for inhibiting class switching than splenic Tfr cells.\textsuperscript{19} Thus, whether cTfr cells can be regarded as an alternative to investigate bona fide Tfr cells needs more validation. cTfr cells might have the capacity to home to secondary lymphoid organs after reactivation\textsuperscript{15,16} and recirculate quickly (about a few hours) through the blood,\textsuperscript{16} whereas their putative remigration and subsequent reactions have not yet studied.

Animal models are generally used to reflect putative human physiology and pathology. Studies by two groups have revealed differences in cTfr function between humans and mice. Sage et al.\textsuperscript{16,18,35,46} showed that cTfr cells in mice can inhibit antibody generation, while Fonseca et al.\textsuperscript{14} reported that cTfr cells in humans are not fully suppressed. Partly because of environmental exposure, murine immune system and immune responses are actually different from those of humans.\textsuperscript{108} From this viewpoint, applications of murine knowledge directly to humans are circumscribed. Furthermore, the origin and TCR repertoire of Tfr cells have been studied only in mice.

Generally speaking, Tfr cells are deemed as repressors of GC reactions, so it is speculated that decreased Tfr/Tfh ratios are associated with enhanced autoantibody generation. Nevertheless, the alterations of Tfr cells in homogeneous diseases are inconsistent and even opposite. Study participants with different stages of the disease and therapeutic regimens may account for part of the reason because methotrexate, a first-line medication for RA, has reduced the frequencies of both Tfr and Tfh cells.\textsuperscript{61} In addition, distinct markers to define Tfr cells in the same anatomic locations and the same markers to define Tfr cells in distinct anatomic locations have brought about great confusion. A possible interpretation for increased Tfr cells in autoantibody-mediated diseases is that it is a compensative response as a result of increased frequency of Tfh cells, attempting to restore immune homeostasis. Another is that suppressive function of Tfr cells is impaired in disease settings; thus, the overall effect of controlling GC responses is still attenuated. In addition, it has also been proposed that the microenvironment while thymic Treg cells differentiate into eTfr cells has altered so that eTfr cells have difficulty in getting access to GCs, leaving them in the peripheral blood.

Also unknown is whether Tfr cells convert from suppressive to stimulatory function in GC responses. Although most studies have demonstrated that Tfr cells function as inhibitors of Tfh and GC B cells, it has been proven that Tfr cells do not influence Tfh or GC B-cell gross population in a Bcl6\textsuperscript{+/F}Foxp3\textsuperscript{cre} mouse model.\textsuperscript{45} Furthermore, Tfr cells help maintain high levels of high-affinity antigen-specific antibodies\textsuperscript{5} and regulate the isotype switch of antibodies.\textsuperscript{45} They also promote the GC response through the secretion of IL-10 and the suppression of cytotoxic Tfh cells.\textsuperscript{53,57} Thus, Tfr cells might play a complicated role in the fine-tuning of GC response and act as ‘helper cells’ in certain scenarios.

Recently, Treg cells have been proven to be not terminally differentiated populations and have
some degree of instability and plasticity under inflammatory conditions, suggesting that Treg cells may lose Foxp3 expression or even acquire the properties of Teff cells. In addition, Teff cells are resistant to suppression by Treg cells in some AIDs. FoXP3 instability in Tfr cells has recently been demonstrated, which results in attenuated suppressive capacity of Tfr cells. The so-called ex-Tfr cells lose their previous transcriptional programme, rendering them more similar to Tfh cells. Considering the shared similarities between Tfr and Treg cells, whether plasticity exists in Tfr cells and whether Tfr and B cells are resistant to Tfr-cell suppression under pathological conditions remain unknown.

Since Tfr cells play such a significant role in regulating antibody production, it is attractive to target Tfr cells to restore immune homeostasis. Altered Tfr/Tfh might be implicated in causes or effects of the above-mentioned diseases. Intraperitoneally injected all-trans retinoic acid in EAMG rats and caspase-1 inhibitor in mice have ameliorated disease severity concomitant with an increased frequency of Tfr cells and decreased frequency of Tfh cells. Baicalin treatment has also been found to ameliorate lupus nephritis in MRL/lpr lupus-prone mice by enhancing the expansion of Tfr cells and suppressing the differentiation of Tfh cells. Methods to enhance Treg cells numerically and functionally have been under clinical trials in AIDs, so we wonder whether Tfr cells can be applied for therapeutic interventions in AIDs, infections, cancers, allergies and other disorders. According to their role in inhibiting antibody generation, regulation of Tfr cells may strengthen the efficacy of vaccines.

**PERSPECTIVES**

The discovery of Tfr cells has provided novel insights into the regulation of humoral immunology, although it is still in the nascent stage. Future emphasis should be put on intricate factors that influence the differentiation and

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**Table 3.** Studies comparing cTfr and GC Tfr cells

| Authors          | Gating                          | Samples                                      | Origin                  | Phenotype                                                                 | Function                                                                 |
|------------------|---------------------------------|----------------------------------------------|-------------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Sage et al.      | CD4+ICOS+CXCR5+Foxp3+GITR+CD19 | Mice immunised with NP-OVA or NP-HEL in CFA  | Primed by DC, do not require B cells | Memory-like, persist in vivo for a long time, express similar levels of CXCR5 but lower ICOS, similar proportions in cell cycle | Similar to LN Tfr cells but with a much lower capacity                    |
| Dhaeze et al.    | CD4+CD25+CD127−CXCR5+PD-1      | Non-AIDs adult patients with routine tonsillectomies | Lymphoid-resident Tfr cells after a GC response | Express lower levels of follicular markers (CXCR5, PD-1, Bcl-6 and ICOS) but similar levels of regulatory markers (Foxp3 and Helios) with comparable Foxp3 methylation status and higher levels of CD31, CCR7 and CD62L, display a memory phenotype and higher percentage of Th1-like phenotype | Comparable suppressive function with tonsil-derived Tfr cells |
| Fonseca et al.   | CXCR5+Foxp3+CD4+CD127−CD127−CD4 | Healthy children with routine tonsillectomies | Peripheral lymphoid tissues before T-B interaction | Naive-like phenotype (high levels of CD45RA, CCR7, CD62L and CD27 and low levels of HLA-DR), CD45RO+Foxp3lo resting cells are the majority, do not express ICOS, PD-1 or Bcl-6 | Able to suppress activation of B cells and proliferation of Tfh cells, do not inhibit class switch recombination |

AIDs, autoimmune diseases; CFA, complete Freund’s adjuvant; cTfr, circulating follicular regulatory T; DC, dendritic cell; GC, germinal centre; HLA-DR, human leucocyte antigen–DR; LN, lymph node; NP-HEL, 4-hydroxy-3-nitrophenylacetyl hapten–conjugated hen egg lysozyme; NP-OVA, 4-hydroxy-3-nitrophenylacetyl hapten–conjugated ovalbumin.
function of Tfr cells. Further elucidation of Tfr cell suppressive mechanisms is essential for Tfr cell–related therapeutics. Tfr cells have been studied in a wide range of diseases, predominantly AIDs, and most of the samples derive from peripheral blood. A major challenge in human research is to obtain lymphoid tissues to assess bona fide Tfr cells. LN fine-needle aspirates (FNAs), a minimally invasive method primarily used for cancer pathology surveillance, may provide access to GCs in humans.113 LN FNAs have already been applied in a pioneering longitudinal study for human GC investigation.114 Undoubtedly, the establishment of a precise definition of Tfr cells in different anatomic locations and relevant animal models is of equal importance. Substantial discrepancies and problems remain to be solved, and these answers will undoubtedly improve immunotherapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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