Foxp3<sup>+</sup> T<sub>reg</sub> cells in humoral immunity

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T<sub>reg</sub> cells are essential for the maintenance of immune homeostasis and prevention of autoimmunity. In humoral immune responses, loss of T<sub>reg</sub> cell function causes increased levels of serum autoantibodies, hyper-IgE, spontaneous generation of germinal centres, and enhanced numbers of specialised T follicular helper cells (T<sub>fh</sub> cells) controlled by the lineage-defining transcription factor BCL-6 (B-cell lymphoma 6). Recent studies have demonstrated that a subset of T<sub>reg</sub> cells (T follicular regulatory (T<sub>reg</sub>) cells) are able to co-opt the follicular T-cell program by gaining expression of BCL-6 and travelling to the follicle where they have an important role in the control of expansion of T<sub>fh</sub> cells and the germinal centre reaction. However, the mechanisms by which they exert this control are still under investigation. In this review, we discuss the effects of T<sub>reg</sub> cells on humoral immunity and the mechanisms by which they exert their regulatory function.

Keywords: antibody, CTLA-4, T-follicular helper cells, T-follicular regulatory cells

Introduction

The generation of high-affinity antibody is critical to protection from infectious disease and other threats. During the T-dependent antibody response, antigen-presenting B cells at the border of the T-cell zone form cognate interactions with T<sub>reg</sub> cells and, following co-stimulatory interactions, may either form short-lived extrafollicular plasmablast cells—responsible for rapid but low-affinity antibody production (1)—or traverse to the B-cell follicle. Once activated in situ there, B cells begin to proliferate and form a germinal centre where longer-lasting, high-affinity responses are induced (2,3).

Although germinal centres are critical for the generation of protective antibody responses, their dysregulation may lead to autoimmunity (4). A large proportion of B cells are autoreactive at an early stage of development (5) and, despite central tolerance mechanisms such as receptor editing, clonal deletion and anergy (6–8), autoreactive B cells are still present in the periphery. Additionally, somatic hypermutation within the germinal centre allows the generation of autoreactive B cells during the response to foreign antigens (9) and also allows chromosomal translocations that have a causative role in the generation of lymphomas (10–12).

As such, it is evident that self-reactive germinal centres must be controlled to avoid autoimmunity but also that some level of regulation of even non-self-reactive germinal centres must be in place. This is achieved by a number of mechanisms such as antibody feedback (13) and follicle-resident CD8<sup>+</sup> T cells (14,15). However, in this article we will focus on the contribution of Foxp3-expressing T<sub>reg</sub> cells to the control of the humoral response. Various phenotypes of T cells have immunosuppressive properties, but the best understood are CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells. These cells are critical to the regulation of humoral immunity as both mice and humans with loss of Foxp3 function have raised levels of serum antibodies (16,17). More recently, it has also become clear that T<sub>reg</sub> cells are able to travel into the B-cell follicle and directly regulate the germinal centre response (18–20).

T<sub>reg</sub> cells

Foxp3<sup>+</sup> T<sub>reg</sub> cells make up around 10% of peripheral CD4<sup>+</sup> T cells and have a critical role in the maintenance of immune self-tolerance and homeostasis (21,22). The function of T<sub>reg</sub> cells is controlled by two key features: (i) the expression of the transcription factor Foxp3 (23–25), responsible for the maintenance of several key phenotypic factors in T<sub>reg</sub>-cell function such as CTLA-4 expression and repression of IL-2 production and (ii) the maintenance of T<sub>reg</sub>-type epigenetics, a specific DNA hypomethylation pattern that is required for the stability and full functional capabilities of T<sub>reg</sub> cells (26). In cases where expression of Foxp3 is lost, such as in the scurfy mouse strain and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients, the resulting loss of T<sub>reg</sub>-cell function causes a range of severe immune disorders such as widespread autoimmunity, immunopathology and lymphoproliferation (17,24,27–29).

In terms of humoral immunity, Foxp3-deficient mice have uncontrolled germinal centre reactions and large numbers of plasma cells and T follicular helper cells (T<sub>fh</sub> cells) (20,30). In the K/BxN mouse model of autoantibody-driven arthritis, loss of T<sub>reg</sub>-cell function, owing to the introduction of the Foxp3 scurfy mutation, leads to accelerated production of...
pathogenic autoantibodies and accumulation of splenic plasma cells (30,31). In humans, B cells from IPEX patients produce large amounts of autoantibody antibodies (32). Another characteristic feature of the loss of Foxp3 function is hyper-IgE in both mice (33) and IPEX patients (29). This may be partly because of the disproportionate effect of the loss of Treg-cell function on IL-4, critical for IgE production, in comparison with other cytokines (34).

Although valuable information can be gained from the study of systems where Treg cells are absent from birth, it can be difficult to determine whether particular phenotypes are specifically the result of Treg-cell depletion or secondary to the high levels of inflammation seen in such circumstances. This issue has been addressed by models in which mammalian diphtheria toxin receptor is co-expressed with Foxp3, making Treg cells sensitive to diphtheria-toxin-induced cell death and allowing specific depletion of Treg cells in adult mice, which in turn leads to the induction of widespread autoreactivity similar to scurfy mice (35,36). The use of anti-CD25 antibodies to deplete Treg cells is also effective.

A number of studies have used these systems to address the consequences of peripheral Treg-cell depletion on the humoral immune response and the generation of autoantibodies. CD25-based depletion of Treg cells leads to increased autoantibody production in a murine model of arthritis (37) or in lupus-prone NZB/NZW F1 mice (38). Accordingly, transfer of ex-vivo-expanded Treg cells into NZB/NZW F1 mice ameliorates disease (39). The maintenance of peripheral anergy in autoreactive B cells is a critical tolerance mechanism, preventing activation of autoreactive B cells (8,40,41). In the absence of Treg cells, normally anergic, dsDNA-specific B cells lose their anergic phenotype and, following provision of T-cell help, produce anti-ds-DNA antibodies. Transfer of CD25+- Treg cells prevents this process (42). Correspondingly, specific Treg-cell depletion via diphtheria toxin also leads to a loss of B-cell anergy (43). Glucocorticoid-induced TNFR-related protein (GITR) is expressed at high levels on Treg cells and is important in their suppressor functions; depletion of GITR-expressing Treg cells also leads to the development of anti-myosin autoantibodies and myocarditis (44).

Since Treg cells are generated in the thymus through selection of high-affinity, self-reactive TCRs (45,46), their TCR repertoire is skewed towards recognition of self-antigens. This raises the question of whether Treg cells will be capable of efficiently recognizing complexes of MHC class II (MHCII) plus foreign peptide or are restricted to control of self-reactive cells. Indeed several studies examining the effects of Treg-cell depletion on autoantibodies have shown no similar effect on foreign antigens (37,47).

Recent work by Lee et al. (48) demonstrates that the thymic selection process that results from Treg cell possession of high-affinity TCRs may be stochastic, rather than a certain TCR signal strength guaranteeing selection of either an effector T cell or a Treg cell. This has been addressed experimentally by the Jenkins group using MHCII tetramers (i.e. specific antigen bound to four MHCII monomers to increase avidity) to demonstrate that Treg cells with foreign-antigen-specific TCRs are present in both the thymus and periphery of naive animals (49). When the same antigen was transgenically expressed, effectively converting it to a ‘neo’ self-antigen, numbers of both Foxp3+ and Foxp3+ tetramer-binding cells were reduced due to clonal deletion. However, Foxp3 cells were more resistant to deletion, ensuring that the ratio of antigen-specific Foxp3+ cells to Foxp3+ cells is higher for self-antigens (49).

As a molecular mechanism of this self-skewing of the Treg TCR repertoire, CTLA-4 expression by developing Treg cells may play a key role. Both CTLA-4 and CD28 on T cells can bind either CD80 (B7-1) or CD86 (B7-2). Binding of CD80 provides co-stimulatory signals to the T cell, but as detailed later, CTLA-4 interferes with this by depletion of CD80 and CD86 from the surface of APCs (50–52). CTLA-4 may thus attenuate the TCR signal to developing Treg cells, allowing self-reactive Treg cells that would otherwise receive a signal sufficiently strong to induce their apoptosis to escape negative selection (53).

Taken together, these findings suggest that Treg cells are able to control both foreign-antigen-reactive and autoantigen-reactive cells but that the relative ratios between antigen-specific Foxp3+ and Foxp3+ T cells are altered, dependent on recognition of foreign or self-antigens. For foreign antigens, there are more Foxp3+ than Foxp3+ Treg cells, whereas for self-antigens, there are more Foxp3+ than Foxp3+ Treg cells. These different ratios mean that Treg cells may tightly control autoreactive responses, while the level of control of foreign reactive cells is less stringent but not non-existent, allowing proper immune responses to pathogens while still maintaining a level of control to prevent excessive responses.

Since the effect on the response to foreign antigens is likely to be more subtle, this may explain why it has not always been observed. Accordingly, it has been demonstrated that loss of Treg cells or T follicular regulatory cells (Treg cells; see below) may enhance humoral immune responses to foreign antigens (18,20,54). Treg cells also have an important protective role in a wide range of infection models (55). For instance, Treg cell depletion enhances the CD8+ T-cell response to pathogens such as herpes simplex virus (56,57) and Listeria monocytogenes (58).

In some cases, Treg-cell effects on pathogen–host interactions may be because of a broader role in damping inflammation; however, the specificity of Treg cells for pathogen epitopes has been demonstrated in several studies. Treg cells that bind MHCII tetramers specific for peptides from influenza nucleoprotein are detectable following influenza infection (59) and their depletion during secondary infection enhances numbers of antigen-specific CD8+ cells, whereas Treg cells recognizing epitopes from the rJ2.2 stain of mouse corona viral disease (60).
co-stimulator ligand (ICOSL) binding T-cell CD28 and ICOS that allows the full commitment and maintenance of the Tfh lineage (66–70). Expression of the chemokine receptor CXCR5, in combination with a loss of CCR7 expression, allows Tfh cells to travel into the B-cell follicle (71). Tfh-cell CD40 ligand (CD40L) expression provides help for B cells and production of various cytokines, notably IL-21, by Tfh cells drives the survival, differentiation and class-switching of B cells (72,73). These actions expand and maintain the germinal centre reaction, allowing the generation of high-affinity antibody and production of long-lasting plasma cells and memory B cells (63,64).

The vital role of Tfh cells in the class-switching of antibody isotypes was underlined by elegant experiments demonstrating that cytokines produced by Tfh cells directly control B-cell isotype class-switching. T-cell–B-cell doublets were isolated from reporter mice producing the relevant cytokines, and it was found that B cells in contact with IL-4-producing Tfh cells had switched to IgG1 production, whereas B cells in contact with IFN-γ-producing Tfh cells had switched to IgG2a production (73). Tfh cells also appear to have a critical role in the generation of IgE because inhibition of Tfh-cell IL-4 production has profound effects on IgE production, whereas loss of T2-cell IL-4 production had little effect (74).

Although it is clear that Tfh cells have a vital role in the development of protective responses to pathogens, their excessive expansion in ICOS-overexpressing mice leads to lupus-like pathology and autoantibody production (75,76). Correspondingly, in humans, large-scale expansion of Tfh cells is associated with severe systemic lupus erythematosus (SLE) (77), demonstrating the need for tight peripheral control of these cells.

Given the role of Tfh cells in the control of humoral immunity, an important question to answer was whether, similarly to Tfh cells, Tfh cells are able to travel into the follicle where they might directly regulate the germinal centre reaction. Pioneering work from Lim and colleagues demonstrated that, upon activation, CD4+CD25+CD69 Tfh cells are able to downregulate CCR7 while simultaneously upregulating CXCR5, allowing them to migrate into the B-cell zone (78). As a result, these Tfh cells could be visualized in the germinal centres of human tonsillar sections (78,79). The Tfh cells are able to suppress T-cell-dependent antibody production as well as directly inhibit antibody production by CD40-stimulated B cells in the absence of Tfh cells (78,79).

More recently, several groups were able to significantly expand on these earlier observations with the discovery that a subset of Tfh cells are able to co-opt the Tfh-cell pathway by gaining expression of the transcription factor BCL-6 (18–20). These Tfh cells are able to travel to the germinal centre and control the expansion of Tfh cells and germinal centre B cells (18–20). Similar to Tfh cells, Tfh cells express high levels of CXCR5, PD-1 (programmed cell death 1) and ICOS, but differ from Tfh cells by a lack of IL-4, IL-21 and CD40L expression, while also expressing high levels of Tfh-cell associated suppressive molecules such as CTLA-4 and IL-10 (19). Tfh cells appear to differentiate from natural Tfh cells (which already have their regulatory phenotype in the thymus), rather than being a form of induced Tfh cells (which develop their regulatory phenotype only in the periphery), and require many of the same signals as Tfh cells as their formation is dependent on CD28, and SAP (signalling lymphocytic activation molecule-associated protein) signalling and interactions with B cells (18–20).

The in vivo role of Tfh cells has been demonstrated by both adoptive transfer and bone marrow chimera of cells from CXCR5, BCL-6 or SAP-knockout (KO) mice. Interestingly, while it is clear that Tfh cells control expansion of Tfh cells and germinal centre B cells, conflicting results were obtained regarding their control of the specific response to a foreign vaccine antigen. It was reported that a loss of Tfh cells leads to an increase in antigen-specific B cells and the resulting antibody response (18,20). This contrasts with the observation that although total numbers of germinal centre B cells increased, there was an overall drop in the number of antigen-specific B cells and the resulting serum antibody levels (19). It was suggested that a large increase in autoreactive cells effectively outcompeted antigen-specific B cells to the extent that antigen-specific antibody was reduced (19).

Earlier studies examining depletion of total Tfh cells have also reported that Tfh-cell depletion may (54) or may not (37,47,80) enhance the responses to foreign antigens. In our hands, we are able to generate both results using different Tfh-cell depletion strategies, suggesting it is essentially a dose-dependent phenomenon.

Thus, we suggest a model in which temporary depletion of Tfh cells leads to activation of antigen-presenting cells (APCs) and increased formation of Tfh cells without disrupting immune homeostasis, allowing antigen-specific cells to expand effectively. However, beyond a certain threshold, homeostasis is disrupted and the overwhelming numbers of antigen-non-specific or autoantigen-specific cells expanding in the absence of Tfh cells may prove deleterious to an antigen-specific response (Fig. 1). Indeed we have found that while short-term depletion of Tfh cells around the time of vaccination leads to enhanced numbers of tetramer-binding Tfh cells and antigen-specific germinal centre B cells, prolonged Tfh-cell depletion leads to high levels of Tfh cells and germinal centre expansion but a reduction in the total number of tetramer-binding Tfh cells, with the percentage of

**Fig. 1.** Model of vaccine-specific and polyvalent responses and correlation with level of Tfh-cell depletion. As Tfh-cell depletion increases, the vaccine-induced response to foreign antigens (blue line) is first enhanced by a loss of antigen-specific Tfh cells and increased CD28 signalling but later, the response is reduced by large-scale expansion of autoreactive cells (red line) competing for resources.
antigen-recognizing B cells dropping in correlation with germinal centre expansion (J. B. Wing et al., in preparation).

Mechanisms of T<sub>reg</sub>-cell modulation of humoral immunity

T<sub>reg</sub> cells have a wide range of suppressive mechanisms that may be available for use in different contexts and dependent on the level of stimuli that individual T<sub>reg</sub> cells have been exposed to (81). A number of mechanisms by which T<sub>reg</sub> cells regulate humoral immunity either by direct contact with B cells or suppression of T-cell help have been proposed, which are summarized below.

CTLA-4

The inhibitory molecule CTLA-4 is highly expressed on the surface of T<sub>reg</sub> cells and plays a critical role in their inhibitory function both in vitro and in vivo by limiting availability of CD80 and CD86 (52,82–85). CD80 and CD86 expressed by APCs provide essential co-stimulatory signals to T cells via ligation of CD28 in addition to TCR signalling (86). T cells from CD28-deficient mice lack expression of CXCR5 and have a reduced ability to differentiate to T<sub>fh</sub> cells (87,88), despite the near-total absence of T<sub>reg</sub> cells (89). As such, CD28-deficient mice also fail to form germinal centres (87,90).

T<sub>reg</sub>-cell-specific conditional KO (cKO) of CTLA-4 leads to the generation of fatal autoimmune and lymphoproliferative disorders similar to those seen in Foxp3-deficient scurfy mice or full CTLA-4 KO mice (52,83,84). Total loss of CTLA-4 expression has profound effects on humoral immunity as effector T cells produce large amounts of IL-4 leading to hyper-IgE production (82). In T<sub>reg</sub>-cell-specific CTLA-4 cKO mice, we have also previously reported hyper-IgE and enhanced IgG in the serum (52). More recently we have observed high levels of spontaneous development of T<sub>fh</sub> cells and germinal centres in CTLA-4 cKO mice, similar to those seen in scurfy mice, suggesting that, in the absence of CTLA-4, other T<sub>reg</sub>-cell effector mechanisms are insufficient to regain control of humoral immunity despite a large number of T<sub>reg</sub>/T<sub>eff</sub> cells (20) (J. B. Wing et al., in preparation).

It is well established that CD80 and CD86 are critical for humoral immune responses and that it is possible to efficiently block their function with a solubilized form of CTLA-4 (CTLA-4–Ig), resulting in drastically inhibited vaccine responses and reduced germinal centre formation (91,92). However, in vivo, the blocking effect of large doses of CTLA-4–Ig may not be an accurate correlate for the in vitro function of membrane-bound CTLA-4 found on T<sub>reg</sub> cells, which appears to act primarily via downregulation of surface expression of CD80/CD86 on DCs and B cells (50,52).

The exact mechanism by which CTLA-4 is able to deplete CD80/CD86 had been sought for some time. However, recent elegant work by Qureshi et al. has shed light on this process by demonstrating that CTLA-4 is able to trans-endocytose CD80/CD86 from the surface of the APC and take it into the T<sub>reg</sub> cell, where it is then degraded (51). In addition to its role in the control of CD80 and CD86 expression, CTLA-4 may also directly inhibit the function of CD80/CD86-expressing DCs by inducing nuclear translocation of the transcription factor Foxo3, leading to a loss of IL-6 expression (93) or modulating tryptophan catabolism by induction of the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) (94).

CTLA-4 binding to B-cell CD80/CD86 may also have a direct effect on antibody production within established germinal centres. Consistent with this notion, putative CD25–CD69– T<sub>reg</sub> cells are able to directly reduce B-cell antibody production in vitro, but this suppressive effect was partially abrogated by the addition of CTLA-4-blocking antibodies (79). However, definitive proof, such as a true understanding of the molecular mechanisms involved in this reverse signalling into B cells, has remained elusive.

As previously noted, CD28-, CD80- or CD86-deficient mice have impaired formation of T<sub>fh</sub> cells (95,96). As such, it seems likely that the key role of CTLA-4 in humoral immunity may be via control of CD80 and CD86 expression by APCs and the resultant effects on T<sub>fh</sub>-cell formation. Interestingly, it seems that B-cell expression of CD80 and CD86 is of particular importance, as addition of CD86-expressing B cells to CD86-KO mice is sufficient to restore formation of T<sub>fh</sub> cells (95); and B-cell-specific expression of CD80 also affects T<sub>fh</sub>-cell generation and subsequent production of plasma cells (96).

Interestingly, given the particular role of both T<sub>reg</sub> cells and CTLA-4 in the control of IgE production, Tian et al. demonstrated that T<sub>reg</sub>-cell depletion has a disproportionate effect on IL-4 production (34). Although they interpreted this as evidence of control of T<sub>fh</sub> cells, more recent evidence suggests that IL-4 produced by T<sub>fh</sub> cells may be critical for control of the IgE response (74). Given that both loss of T<sub>reg</sub>-cell function and T<sub>reg</sub>-cell-specific expression of CTLA-4 have a profound effect on the formation of T<sub>fh</sub> cells, it seems likely that this characteristic hyper-IgE seen in T<sub>reg</sub>-cell-deficient mice and IPEX patients (29,33) may be due to the loss of T<sub>reg</sub>-cell CTLA-4-dependent control of T<sub>fh</sub>-cell expansion and IL-4 production.

PD-1

The inhibitory receptor PD-1 is part of the same superfamily as CD28 and CTLA-4 and is believed to be important in the regulation of a number of immune pathways (97). PD-1 is upregulated following activation of B cells, T cells and myeloid cells and notably, is highly upregulated on both T<sub>reg</sub> cells and T<sub>eff</sub> cells, suggesting that it may have a role in the function of these cells (19,20,70).

PD-1 has been implicated in the control of humoral immunity. Total KO of PD-1 leads to dysregulation of humoral immunity in several settings. In BALB/c mice, anti-parietal autoantibodies and anti-cardiac tropon I autoantibodies are produced (98,99), and in C57BL/6 mice, development of lupus-like disease and arthritis occurs, while anti-nuclear antibody levels are enhanced in autoimmune-prone FcγRIII-deficient mice (100). This increase in autoimmunity and autoantibody production may be because of the observed expansion of T<sub>fh</sub> cells in either PD-1-deficient mice or following PD-1 blocking (101,102). However, it seems that this dysregulation also results in a loss of quality as the generated T<sub>fh</sub> cells may have reduced IL-4 and IL-21 expression leading to reductions in the numbers of long-lasting plasma cells (103) and the production of large amounts of low-affinity IgA in the Peyer’s patches that fail to properly control the gut microbiota (104).
More recently, PD-1 expression has also been demonstrated to be critical to maintaining normal T<sub>reg</sub>-cell function. In the absence of PD-1, T<sub>reg</sub> cells have increased suppressive potential and have an enhanced ability to inhibit antibody production both in vitro and in vivo, presumably because the lack of PD-1 cell-intrinsic inhibitory signals allows the cells to become more highly activated (105). It seems likely that since T<sub>reg</sub> cells and T<sub>H</sub> cells are present in the B-cell follicle where stimulatory signals may be in excess, it is necessary for them to express inhibitory receptors such as PD-1 to prevent their overactivation, leading to either autoimmunity or excessive suppression of the immune response for T<sub>reg</sub> cells and T<sub>H</sub> cells.

Expression of PD-1 ligands on T<sub>reg</sub> cells may also play a role in their suppressive function. Recently Gotot and colleagues demonstrated that T<sub>reg</sub>-cell-specific expression of PD-1 ligand 1 and PD-1 ligand 2 directly inhibits the function of autoreactive, PD-1-expressing B cells without the need for intermediate interactions with T<sub>H</sub> cells (106).

**IL-10**

The pleotropic cytokine IL-10 has a complex role in humoral immunity, having functions that may either inhibit or enhance the antibody response. In vitro, addition of exogenous IL-10 to IL-4-stimulated human PBMCs suppresses IgE production and enhances IgG4 production (107,108) while also acting as a switching factor for human IgG1 and IgG3 (109). In addition, IL-10 stimulation of B-cells isolated from SLE patients, but not healthy donors, enhances their production of IgM, IgA and IgG, demonstrating that in certain pathological circumstances, IL-10 may be capable of directly enhancing auto-antibody production (110). Furthermore, following stimulation with IL-10, ger- minal centre B cells upregulate Bcl-2 expression, preventing their apoptosis (111), although this does not appear to be the mechanism of enhanced antibody production from B-cells isolated from lupus patients (110). On the other hand, IL-10 down-regulates the inflammatory function of macrophages and DCs (112), a notion that is implicated in MRL-Fas<sup>Δκ</sup> IL-10-deficient mice which suffer an increased severity of lupus (113).

Total KO of IL-10 leads to inflammation in the gut mucosa (114). However, it is difficult to be certain to what extent results from total IL-10 KO mice are due to a lack of T<sub>reg</sub>-cell IL-10 production and not IL-10 production by other cells, notably other described immunoregulatory subsets such as Foxp3<sup>CD4+</sup> IL-10-producing Tr1 cells and IL-10-producing B cells (115,116). Interestingly, IL-10R<sup>KO</sup> mice also have quantitative increases in CXCR5<sup>+</sup> T-cell formation following vaccination in addition to qualitatively increases in IL-21 and IL-17 expression by said cells (117). However, since T<sub>reg</sub> cells themselves appear to require IL-10 signalling to maintain their full function and control of IL-17-producing cells, the possibility of reduced IL-10 signalling inhibiting T<sub>reg</sub>-cell function must be also be considered (118). Also, purified DCs from IL-10R<sup>KO</sup> KO mice had enhanced IL-6 and IL-23 expression following in vitro stimulation, again demonstrating a cell-intrinsic role in suppressing DC function (117).

IL-10 is not critical to the in vitro suppressive function of T<sub>reg</sub> cells (119,120). However, similar to full IL-10 KO mice, cKO of IL-10 in T<sub>reg</sub> cells leads to the development of colitis but not the systemic autoimmunity seen in Foxp3<sup>-</sup> or CTLA-4-deficient mice (120). The colitis does not seem to be primarily driven by autoantibodies but instead by IL-17-producing T<sub>17</sub> cells (121). As such, it appears that T<sub>reg</sub>-cell-expressed IL-10 is most critical at maintaining immune homeostasis at the mucosal surfaces.

The role of IL-10 in humoral immunity is complex, since B cells, DCs, effector T cells and T<sub>reg</sub> cells all express IL-10R<sup>α</sup> and can also produce IL-10 itself. Although there is currently little direct evidence that IL-10 produced specifically by Foxp3-expressing T<sub>reg</sub> cells is critical to the control of humoral immunity, it seems likely that given the ability of IL-10 to regulate DC function and T<sub>H</sub>-cell differentiation while also directly inhibiting production of at least some subclasses of antibody, particularly IgE T<sub>reg</sub>-cell production of IL-10 may have some role in the control of antibody production. Importantly, T<sub>reg</sub> cells have been reported to have increased levels of IL-10 mRNA expression (19), suggesting that this may play some role in their suppressive function within the B-cell follicle.

**TGFβ**

The suppressive cytokine TGFβ has also been implicated in T<sub>reg</sub>-cell suppression of antibody responses. Membrane-bound, but not soluble, TGFβ is able to mediate contact-dependent suppression of B-cell antibody production (122). Additionally, in vitro treatment of T<sub>reg</sub> cells with anti-TGFβ antibody has been reported to suppress B-cell antibody production, either by direct action on the B cells themselves (79) or via inhibition of T-cell help (54).

However, the role of TGFβ may be more complex than simple suppression of antibody responses, since it is also able to induce isotype-switching to IgA (123). As such, T<sub>reg</sub>-cell TGFβ production has been demonstrated to enhance the induction of IgA production in the mucosa (124).

**Induction of B-cell apoptosis**

A particularly direct mechanism by which T<sub>reg</sub> cells may control B-cell responses is by induction of their apoptosis. In vitro, activated T<sub>reg</sub> cells preferentially kill antigen-presenting B cells rather than bystander cells. Similarly, T<sub>reg</sub> cells from both human sufferers of SLE and lupus-prone NZB/NZW F<sub>1</sub> mice also induce B-cell apoptosis via granzyme and perforin (47,125,126). In addition to granzyme-based induction of apoptosis, Fas–FasL-induced lysis of B cells by T<sub>reg</sub> cells may occur (127). Interestingly Fas–FasL interactions have also been recently demonstrated to be critical to the maintenance of germinal centre homeostasis (128). Currently no information on the role of apoptosis induction by T<sub>reg</sub> cells is available, but T<sub>reg</sub> cells have been reported to have decreased levels of granzyme B mRNA expression, suggesting that this particular pathway may not be critical to their function (19).

**Conclusions**

T<sub>reg</sub> cells have a vital role in the control of T-dependent antibody responses. Given their self-antigen-skewed TCR repertoire, it seems likely that T<sub>reg</sub> cells should preferentially control expansion of autoreactive T- and B-cell...
clones. However, sufficient evidence exists to suggest that T\(_{\text{reg}}\) cells may also be important for the control of non-self-responses.

It is clear from the many proposed mechanisms that T\(_{\text{reg}}\) cell modulation of humoral immunity is a complex process, likely to involve several different mechanisms acting either synergistically or redundantly. Given its clear effects on antibody production, we suggest that CTLA-4 is the core mechanism of T\(_{\text{reg}}\)-cell function controlling the formation of germinal centres. However, T\(_{\text{reg}}\) cells, similar to T\(_{\text{reg}}\) cells from other sites where stimulatory signals are in excess (81), may utilize a range of other suppressive mechanisms such as IL-10 and TGF\(\beta\), thereby allowing them to play an important role in the fine-tuning of established germinal centre responses (Fig. 2).

Polyclonal depletion of T\(_{\text{reg}}\) cells is a valuable tool that has allowed us to assess the function of T\(_{\text{reg}}\) cells in humoral immunity. However, because of the role of T\(_{\text{reg}}\) cells in the maintenance of immune homeostasis, it is perhaps too blunt a tool to allow fine modulation of antigen-specific responses, due to the possibility of inhibiting the antigen-specific response following excessive expansion of polyclonal cells and the inherent risk of autoimmune side effects. In the future, it will be imperative to develop new methods that allow the preferential depletion or expansion of antigen-specific T\(_{\text{reg}}\) cells following vaccination, allowing the enhancement or negation of antigen-specific responses while maintaining immune homeostasis by preserving the polyclonal T\(_{\text{reg}}\)-cell repertoire.

**Fig. 2.** Mechanisms of T\(_{\text{reg}}\)-cell suppression. During the events of germinal centre (GC) formation, T\(_{\text{reg}}\) cells acting outside the follicle, possibly at the T–B border, act to regulate CD28 co-stimulation via CTLA-4, while also inducing apoptosis of specific B cells via granzyme. Within the GC, it is likely that T\(_{\text{reg}}\) cells use a number of mechanisms such as IL-10 and TGF\(\beta\) in addition to CTLA-4.

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**References**

1. McHeyzer-Williams, L. J., Malherbe, L. P. and McHeyzer-Williams, M. G. 2006. Checkpoints in memory B-cell evolution. *Immunol. Rev.* 211:255.
2. Klein, U. and Dalla-Favera, R. 2008. Germinal centres: role in B-cell physiology and malignancy. *Nat. Rev. Immunol.* 8:22.
3. MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117.
4. Vinuesa, C. G., Sanz, I. and Cook, M. C. 2009. Dysregulation of germinal centres in autoimmune disease. *Nat. Rev. Immunol.* 9:845.
5. Wardemann, H., Yurasov, S., Schaefer, A., Young, J. W., Melfre, E. and Nussenzweig, M. C. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301:1374.
6. Nemazee, D. and Buerki, K. 1989. Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras. *Proc. Natl Acad. Sci. USA* 86:8039.
7. Nemazee, D. 2006. Receptor editing in lymphocyte development and central tolerance. *Nat. Rev. Immunol.* 6:728.
Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance.
Nature 467:328.

15 Noble, A., Zhao, Z. S. and Cantor, H. 1998. Suppression of immune responses by CD8 cells. II. Qa-1 on activated B cells stimulates CD8 cell suppression of T helper 2 responses. J. Immunol. 160:566.

16 Godfrey, V. L., Wilkinson, J. E. and Russell, L. B. 1991. X-linked lymphoproliferative disease in the scurfy (sf) mutant mouse. Am. J. Pathol. 138:1379.

17 Wildin, R. S., Ramsdell, F., Peake, J. et al. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat. Genet. 27:18.

18 Wollenberg, I., Agua-Doce, A., Hernandez, A. et al. 2011. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J. Immunol. 187:4553.

19 Linterman, M. A., Pierson, W., Lee, S. K. et al. 2011. Foxp3+ follicular regulatory T cells control the germinal center response. Nat. Med. 17:975.

20 Chung, Y., Tanaka, S., Chu, F. et al. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat. Med. 17:983.

21 Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M. 2008. Regulatory T cells and immune tolerance. Cell 133:775.

22 Wing, K. and Sakaguchi, S. Regulatory T cells exert checks and balances on self tolerance and autoimmune. Nat. Immunol. 11:7.

23 Hsieh, C. S., Liang, Y., Tyznik, A. J., Self, S. G., Liggitt, D. and Jordan, M. S. 2011. A broad range of self-reactivity drives thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat. Immunol. 2:301.

24 Hsieh, C. S., Liang, Y., Tzytnik, A. J., Self, S. G., Liggitt, D. and Rudensky, A. Y. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity 21:267.

25 Jordan, M. S., Boesteanu, A., Reed, A. J. et al. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat. Immunol. 2:301.

26 Lee, H. M., Bautista, J. L., Scott-Browne, J. and Mohan, J. F. 2006. Suppression of autoimmune myocarditis and multiorgan inflammation by glucocorticoid-induced TNF receptor family-related protein(high), Foxp3-expressing CD25+ and CD25- regulatory T cells. J. Immunol. 176:4748.

27 Hsieh, C. S., Liang, Y., Tzytnik, A. J., Self, S. G., Liggitt, D. and Rudensky, A. Y. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity 21:267.

28 Ludwig-Portugall, I., Hamilton-Williams, E. E., Gottschalk, C. and Kunts, C. 2008. Cutting edge: CD25+ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens. J. Immunol. 181:4447.

29 Moon, J. J., Dash, P., Oquin, T. H., third al. 2011. Quantitative impact of thymic selection on Foxp3+ and Foxp3- subsets of self-peptide/MHC class II-specific CD4+ T cells. Proc. Natl Acad. Sci. USA 108:14602.

30 Onishi, Y., Fehervari, Z., Yamaguchi, T. and Sakaguchi, S. 2008. Foxp3(+) natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc. Natl Acad. Sci. USA 105:10113.

31 Qureshi, O. S., Zheng, Y., Nakamura, K. et al. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 332:600.

32 Wing, K., Onishi, Y., Prieto-Martín, P. et al. 2008. CTLA-4 control over Foxp3(+) regulatory T cell function. Science 322:271.
73 Reinhart, R. L., Liang, H. E. and Locksley, R. M. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. Nat. Immunol. 10:385.

74 Harada, Y., Tanaka, S., Motomura, Y. et al. 2012. The 3’ enhancer CNS2 is a critical regulator of interleukin-17-mediated humoral immunity in follicular helper T cells. Immunity 36:188.

75 Linterman, M. A., Rigby, R. J., Wong, R. K. et al. 2009. Follicular helper T cells are required for systemic autoimmunity. J. Exp. Med. 206:561.

76 Vinuesa, C. G., Cook, M. C., Angelucci, C. et al. 2005. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature 433:462.

77 Simpson, N., Gatenby, P. A., Wilson, A. et al. 2010. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis Rheum. 62:234.

78 Lim, H. W., Hillsamer, P. and Kim, C. H. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J. Clin. Invest. 114:1640.

79 Lim, H. W., Hillsamer, P., Banham, A. H. and Kim, C. H. 2005. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. J. Immunol. 175:4180.

80 Ludwig-Portugall, J., Hamilton-Williams, E. E., Gotot, J. and Kurts, C. 2009. CD25+ T(reg) cells specifically suppress auto-Ab generation against pancreatic tissue autoantigens. Eur. J. Immunol. 39:225.

81 Yamaguchi, T., Wing, J. B. and Sakaguchi, S. 2011. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. Semin. Immunol. 23:424.

82 Bour-Jordan, H., Grogan, J. L., Tang, Q., Auger, J. A., Locksley, R. M. and Bluestone, J. A. 2003. CTLA-4 regulates the requirement for cytokine-induced signals in T(H)2 lineage commitment. Nat. Immunol. 4:182.

83 Waterhouse, P., Penninger, J. M., Timms, E. et al. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Cdc4-4. Science 270:985.

84 Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A. and Sharpe, A. H. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 3:541.

85 Walker, L. S. 2013. Treg and CTLA-4: Two intertwining pathways to immune tolerance. J. Autoimmun. 45:49.

86 Lenschow, D. J., Walunas, T. L. and Bluestone, J. A. 1996. CD28/ B7 system of T cell costimulation. Annu. Rev. Immunol. 14:233.

87 Linterman, M. A., Rigby, R. J., Wong, R. et al. 2009. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. Immunity 30:228.

88 Walker, L. S., Gulbranson-Judge, A., Flynn, S. et al. 1999. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. J. Exp. Med. 190:1115.

89 Salomon, B., Lenschow, D. J., Rhee, L. et al. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12:431.

90 Ferguson, S. E., Han, S., Kelsoe, G. and Thompson, C. B. 1996. CD28 is required for germinal center formation. J. Immunol. 156:4576.

91 Linsley, P. S., Wallace, P. M., Johnson, J. et al. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4-T cell activation molecule. J. Exp. Med. 176:2057.

92 Walker, L. S., Wiggett, H. E., Gaspal, F. M. et al. 2003. Established T cell-driven germinal center B cell proliferation is independent of CD28 signaling but is tightly regulated through CTLA-4. J. Immunol. 170:91.

93 Dejean, A. S., Beisner, D. R., Ch'en, I. L. et al. 2006. CD4+ CD25+ regulatory T cells control the phenotype that identifies a subset of severe systemic lupus erythematosus. Annu. Rev. Immunol. 24:457.

94 Grohmann, U., Orabona, C., Fallarino, F. et al. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat. Immunol. 3:1097.

95 Salek-Ardakani, S., Choi, Y. S., Rafi-El-Idrissi Benhnia, M. et al. 2011. B cell-specific expression of B7-2 is required for follicular T cell function in response to vaccinia virus. Nat. Immunol. 12:705.
Role of interleukin 10 in the B lymphocyte hyperactivity and spontaneous death of germinal center B cells by induction of the bcl-2 protein. J. Clin. Invest. 93:424.

Banchereau, J., Pascual, V. and O’Garra, A. 2012. From IL-2 to IL-7: the expanding spectrum of anti-inflammatory cytokines. Nat. Immunol. 13:925.

Yin, Z., Baihiy, G., Zhang, N. et al. 2002. IL-10 regulates murine lupus. J. Immunol. 169:2148.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75:263.

Pot, C., Apetoh, L. and Kuchroo, V. K. 2011. Type 1 regulatory T cells (T1) in autoimmunity. Semin. Immunol. 23:202.

Mauri, C. and Bosma, A. 2012. Immune regulatory function of B cells. Annu. Rev. Immunol. 30:221.

Cai, G., Nie, X., Zhang, W. et al. 2012. A regulatory role for IL-10 receptor signaling in development and B cell help of T follicular helper cells in mice. J. Immunol. 189:1294.

Chaudhry, A., Samstein, R. M., Treuting, P. et al. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 34:566.

Takahashi, T., Kuniyasu, Y., Toda, M. et al. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int. Immunol. 10:1969.

Rubtsov, Y. P., Rasmussen, J. P., Chi, E. Y. et al. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28:546.

Huber, S., Gagliani, N., Esplugues, E. et al. 2011. Th17 cells express interleukin-10 receptor and are controlled by Foxp3 and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 34:554.

Nakamura, K., Kitani, A. and Stober, W. 2001. Cell contact-dependent immunosuppression by CD4(+)/CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J. Exp. Med. 194:629.

Coffman, R. L., Leibman, D. A. and Shrade, B. 1989. Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J. Exp. Med. 170:1039.

Cong, Y., Feng, T., Fujihashi, K., Schoeb, T. R. and Elson, C. O. 2009. A dominant, coordinated regulatory cell-IgA response to the intestinal microbiota. Proc. Natl Acad. Sci. USA 106:19256.

Zhao, D. M., Thornton, A. M., DiPaolo, R. J. and Shevach, E. M. 2006. Activated CD4(+)/CD25(+) T cells selectively kill B lymphocytes. Blood 107:3925.

Ikurn, N., Lourenco, E. V., Hahn, B. H. and La Cava, A. 2009. Cutting edge: Regulatory T cells directly suppress B cells in systemic lupus erythematosus. J. Immunol. 183:1518.

Janssens, W., Carlier, V., Vu, B., VanderElst, L., Jacqueline, M. G. and Saint-Remy, J. M. 2003. CD4+CD25+ T cells lyse antigen-presenting B cells by Fas-Fas ligand interaction in an epitope-specific manner. J. Immunol. 171:4604.

Hao, Z., Duncan, G. S., Seagal, J. et al. 2008. Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. Immunity 29:615.