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Inflammation-associated genes: risks and benefits to Foxp3$^+$ regulatory T-cell function

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Summary

Foxp3$^+$ regulatory T (Treg) cells prevent the development of autoimmunity and immunopathology, as well as maintaining homeostasis and tolerance to commensal microorganisms. The suppressive activity of Treg cells is their defining characteristic, generating great interest in their therapeutic potential. However, suppressive and effector functions are not entirely exclusive. Considerable evidence points to the ability of supposedly anti-inflammatory Foxp3-expressing Treg cells to also express transcription factors that have been characterized as cardinal drivers of T effector cell function. We will consider the mounting evidence that Treg cells can function in non-suppressive capacities and review the impetus for this functional change, its relevance to developing immune and autoimmune responses and its significance to the development of Treg-based therapies.

Keywords: autoimmunity; cytokine receptors; cytokines; Foxp3; regulatory T cells; transcription factors.

Introduction

Classification of CD4$^+$ T cells, according to cytokine production and gene expression, defines distinct subsets but struggles to convey the complex and dynamic nature of T-cell function. Polarization of responses is such a central concept that the very language of T-cell biology is built around it. In 1986 Mosmann, Coffman and colleagues classified T helper cell clones according to their distinct patterns of cytokine secretion, and coined the terms Th1 and Th2 establishing the basis for classification as distinct or non-overlapping function.[1] This framework fulfils the basic desire of most biologists to bring order to the world armed only with a dichotomous key and relies on exclusivity to determine position: a Th1 cell, for example, should produce interferon-γ (IFN-γ) but not interleukin-4 (IL-4), whereas the polar opposite is true of a Th2 cell. Newly described T helper cell subsets have joined this exclusive club and distinct patterns of cytokine secretion, under the direction of a subset-specific master regulator of transcription, remain the reference standard for membership. Master-regulators of transcription have been identified for Th1 (T-bet), Th2 (Gata3), Th17 (ROR-γT), follicular helper T (Bcl6) and regulatory T (Treg; Foxp3) cells and they promote lineage commitment by driving the expression of subset-signature genes and repressing genes associated with alternative fates.[2] However, while the defining feature of Th1 and Th2 subsets was their distinction, Th17 cells can be induced to co-express T-bet and ROR-γT and produce both IL-17 and IFN-γ, blurring the lines between Th1 and Th17 function.[3–5] The functional plasticity of Th17 cells redefined our understanding of T-cell subsets, and requires a more fluid framework able to accommodate dynamic changes in transcription factor expression, cytokine production and inflammatory potential in response to environmental conditions. Such plasticity is not restricted to T helper subsets. Treg cells co-expressing effector-associated transcription factors along with Foxp3 and capable of producing pro-inflammatory cytokines are also found in mice and humans (Table 1). What is the purpose of such multifunctional cells and what are the implications for Treg cells in the control of homeostasis, inflammation and therapy?

Co-expression of effector-associated transcription factors

ROR-γT and IL-17

The idea of Treg cells producing effector cytokines runs contrary to their perceived raison d'être. The noble mission statement of Treg cells 'to prevent autoimmunity, immunopathology and maintain homeostasis' is so often

Table 1. Factors affecting pro-inflammatory features of Treg cells

Species	Transcription factor	Effector cytokine	Phenotype	Switch factor	Stabilizing factor	Suppressive capacity	Accumulation at inflammatory sites	Disease associations
Mouse	ROR-γT [10–13,80,93,95,97,100,101]	IL-17 [10–13,54,55,59,81,95,97,101–104]	CCR6+[81] CCL20+IL-23R+[13] CD44hi[55]	IL-6 IL-1 [10] IL-4[59] STAT3[10,102] TCR [93] IL-1b [100]	IL-2[10,13,54,80] TGF-β[12,59] IDO [93,95] Eos [93] IL-10 [101,103] SOCS-1[103] Ubc13 [103] ATRA [104]	Lost [13,54,95] Retained [93]	Yes [13,54,55,101]	CIA [13] Diabetes [55] GVHD [102] Colitis [81] GI Polyposis [97,101]
Human	ROR-γT [14–18,97,105,106]	IL-17 [14–18,28,53,97,105–107]	CD45RA-HLA-DR-[28] CCR6+[14–18] CCR4+[18] CD161-[18] IL-1R1+[53] CCR7+[53]	IL-1β[14–17] IL-23 [14,17,106] IL-21[14,16] IL-2 [107] TGF-b[16,17] IL-6 [15] Mast cells [105]	IL-1Ra[14]	Lost [107] Retained [15,16,97,105] Retained in IL-1R1+ cells [17]	Yes [13,106]	Colon cancer [97,105] Psoriasis [106] RA[13]
Mouse	T-bet [19,20,23,25–27,38,56,57]	IFN-γ [19,20,23,25–27,54–56,59]	CXCR3+ [19,26,27,38,57]	IFN-γ [19,27,108] IL-12[25,27] IL-27[26] STAT-1 [19]	IL-2 [20,54,56,59]	Retained [19,20,23,27]	Yes [19,20,23,26,27,38,55–57]	Mycobacterial infection [19] Listeriosis [27,57] Toxoplasmosis [20,26] Coronavirus infection [23] EAE [23,38,56] Diabetes [55]
Human	T-bet [21,109]	IFN-γ [21,22,28]	CXCR3+ [21,109]	IL-12 [21,22]		Retained [21,109] Reversibly reduced [22]	Yes [109]	Type 1 diabetes [21] GVHD [28] Multiple sclerosis [22] Ovarian cancer [109]

cited that it overshadows alternative functions. Perhaps Treg multi-functionality should not come as such a surprise. Transforming growth factor-β (TGF-β) promotes differentiation of both Treg and Th17 cells and the decision between these alternative fates is influenced by the prevailing cytokine milieu: IL-6 promotes Th17 development via activation of signal transducer and activator of transcription 3 (STAT3), whereas IL-2-driven activation of STAT5 favours Treg cell induction.[6–9] Foxp3 and ROR-γT are co-expressed during the polarization of Th17 cells, but IL-6 promotes down-regulation of Foxp3 and, in concert with IL-1, drives Th17 differentiation.[10] Both IL-6 and IL-1β can also induce IL-17 production in Treg cells[10,11] (Table. 1).

In vivo, high frequencies of Foxp3$^+$ ROR-γT$^+$ Treg cells are found in the lamina propria, suggesting that they may have a role in maintaining immunological homeostasis.[12] They are also found at sites of ongoing inflammation. In collagen-induced arthritis for example, IL-6 produced by synovial fibroblasts promotes loss of Foxp3 and production of IL-17 by Treg cells. Furthermore, these IL-17-producing Treg cells are capable of driving autoimmunity on transfer.[13] Foxp3$^+$ ROR-γT$^+$ T cells are present in human peripheral blood[14–18] and are enriched in the synovia of patients with active rheumatoid arthritis, suggesting that IL-17 produced by Treg cells may contribute to inflammation.[13]

T-bet and IFN-γ

Th1-like Treg cells are also found in both mice and humans.[19–22] Elevated frequencies of Treg cells expressing T-bet and capable of producing IFN-γ are seen during strongly polarized Th1 responses.[19,20,23] Patients with chronic inflammatory conditions, including relapsing–remitting multiple sclerosis,[22] type 1 diabetes[21] and graft-versus-host disease[24] also have an increased prevalence of CXCR3$^+$ T-bet$^+$ IFN-γ^+ Treg cells. However, elevated T-bet expression does not necessarily limit suppressive activity. During coronavirus infection for example, viral-specific Th1-like Treg cells produce IFN-γ but retain potent antigen-specific suppressive capacity.[23] Similarly, IFN-γ^+ Treg cells in patients with type 1 diabetes retain their suppressive activity *in vitro*.[21] IFN-γ^+ Treg cells from patients with multiple sclerosis show reduced suppressive potential that can be restored by neutralizing IFN-γ or IL-12. Hence, targeting Th1-associated cytokines may increase Treg function as well as having direct anti-inflammatory effects.[22]

The Th1-like characteristics can be induced in Treg cells by the Th1-associated cytokines IFN-γ, IL-12 and IL-27[19,25,26] (Table 1). Interferon-γ activates STAT1, which promotes expression of T-bet and of IL-12rB2, thereby increasing sensitivity to IL-12.[19,25,27] However, in comparison to conventional T cells, IL-12rB2 expression is slower and lower in Treg cells, limiting STAT4 activation and preventing full acquisition of a Th1 effector phenotype.[27] Importantly, the amount of either IFN-γ or IL-17 produced by Treg cells is typically lower than seen in their conventional Th17 and Th1 counterparts, indicating that cytokine production is still restrained in these cells compared with bona fide effector T (Teff) cells.[27,28] Because of this, it is difficult to determine the relevance of the relatively small amounts of pro-inflammatory cytokine produced by Treg cells to the progression of inflammation (see below).

Gata3

Th1-associated or Th17-associated cytokines can induce expression of T-bet or ROR-γT in Treg cells and drive production of effector cytokines. In contrast, the expression of Gata3 in Treg cells is neither induced by Th2-associated cytokines, nor normally associated with elevated Th2-cytokine production by Treg cells. Gata3 is expressed at higher levels in Treg cells than naive T cells in the steady state[29] and promotes Foxp3 expression.[29] Indeed, Gata3 has an essential role in Treg function, Gata3 deficient Treg cells are unable to prevent the development of T-cell-mediated colitis and Treg-specific deletion of Gata3 results in spontaneous autoimmune disease.[29,30] During both infection and autoimmunity Gata3 supports and maintains Treg function at diverse inflammatory sites[29,30] whereas in the steady state Gata3$^+$ Treg cells are prevalent in gut-associated lymphoid tissues, suggesting an important role in homeostasis at barrier sites.[30] By interacting directly with Foxp3, Gata3 can suppress ROR-γ expression and IL-17 production,[29–31] thereby restraining the pro-inflammatory potential of Treg cells (see below). Gata3 expression in Treg cells is induced by T-cell receptor (TCR) activation and IL-2, independently of STAT6 and IL-4/IL-13.[30] Therefore, unlike T-bet or ROR-γT, Gata3 is not induced in response to effector cytokines but by the survival factor IL-2.[30] This may underpin both the general importance of Gata3 to Treg function and the ability of IL-2 to prevent production of IL-17 by Treg cells (see Table 1).

Compared with IFN-γ and IL-17, evidence in the literature for Treg cells producing Th2-associated cytokines is relatively sparse. Production of IL-4 and IL-13 by Treg cells has been associated with the Th2 response driven by respiratory syncytial virus.[32] After repeated infections, Treg cells in the draining lymph node also showed lower suppressive capacity and elevated expression of Gata3.[32] Notably, however, Gata3 expression alone is not sufficient to drive Th2 cytokine production in Treg cells as Gata3$^+$ Treg cells from uninfected mice do not produce effector cytokines.[32] Most recently the first evidence of human Treg cells producing IL-4 and IL-13 has emerged from study of skin samples from patients with the autoimmune

disease systemic sclerosis. Treg cells producing Th2 cytokines remained absent from peripheral blood but a higher proportion of Treg cells isolated from skin biopsies from systemic sclerosis patients produced IL-13 and IL-4 than in the skin of healthy control subjects.[33] These studies emphasize the need to study the behaviour of Treg cells in tissues and at inflammatory interfaces, and show that Treg cells have the potential to produce cytokines, which may contribute to fibrotic as well as acute inflammation.

Treg specialization: fine tuning the Treg response

The current paradigm suggests that Treg cells co-expressing effector associated transcription factors exist to facilitate efficient control of the related effector responses (reviewed in ref. 34). The model proposes that more efficient homing (as a result of a shared profile of chemokine receptor expression between Treg and effector cells) or invocation of subset-specific suppressive mechanisms act to optimize regulation.

Control of Th1 responses

The concept of tailoring Treg activity to the dominant contemporaneous Teff cell response was developed in studies investigating the role of T-bet in Treg cells. An essential role for T-bet in Treg function is not immediately apparent; T-bet-deficient Treg cells show no functional defect in *in vitro* suppression assays.[35,36] T-bet-deficient mice do not have an altered frequency of Treg cells,[35,36] nor do they develop spontaneous autoimmunity or an exacerbated form of induced organ-specific disease.[37] This all suggests that T-bet expression is not required for Treg function. However, in models of Th1 inflammation (anti-CD40 treatment, or infection with *Mycobacterium tuberculosis*[19]) T-bet-deficient Treg cells compete inefficiently with their wild-type counterparts in terms of proliferation and access to inflamed tissue. Hence, in chimeric mice (harbouring both wild-type and T-bet-deficient T cells) with mycobacterial infection, T-bet-deficient Treg cells are disadvantaged in their capacity to home to the inflamed lung. The diminished migratory potential of T-bet-deficient Treg cells results from their lack of CXCR3 expression, which renders them unable to respond to CXCL10 induced by IFN-γ during infection.[19] Once Treg cells have gained entry to the inflammatory site, IFN-γ can increase their IL-10 production, thereby further enhancing their suppressive capacity.[27]

It is important to note that inflammation can drive expression of T-bet and CXCR3 by Treg cells without this being critical to their function. For example, in experimental autoimmune encephalomyelitis (EAE), the Treg cells recruited to the inflamed central nervous system (CNS) have elevated expression of T-bet and are uniformly CXCR3$^+$.[38] However, T-bet-deficient Treg cells are unimpaired in their ability to home to the CNS, demonstrating that alternative, or compensatory mechanisms can efficiently recruit Treg cells in the absence of T-bet.[37] More strikingly, Treg recruitment is unimpaired when T-bet deficiency is restricted to Treg cells and inflammation is driven by highly polarized wild-type Th1 cells.[36] Treg cells constrain production of IFN-γ following immunization.[39] However, mice with T-bet-deficient Treg cells do not develop exuberant Th1 responses after immunization, indicating that T-bet expression by Treg cells is not essential to regulate the induction phase of Th1 responses.[36] Therefore T-bet expression can enhance, but is not necessary for, Treg cell recruitment to sites of Th1-mediated inflammation.

Control of Th2 responses

Treg-specific deletion of the Th2-associated transcription factor interferon regulatory factor 4 (IRF4) leads to development of a lymphoproliferative disease dominated by excessive Th2 responses, rather than the Th1-dominated inflammation typically seen in scurfy mice.[40,41] These results strongly suggest that IRF4 expression promotes effective control of Th2 responses. However, it should be noted that IRF4 is expressed in all activated Treg cells and that, in concert with BLIMP-1, it drives development of an 'effector-Treg' phenotype and expression of IL-10,[42] a cytokine also capable of suppressing both Th1 and Th17 responses.

Control of Th17 responses

Mice lacking STAT3 specifically in Treg cells develop fatal, Th17-dominated colitis.[43] STAT3 is critical for Th17 differentiation[9] and the inability of STAT3$^{-/-}$ Treg cells to control Th17 responses provides a clear parallel with the inability of T-bet$^{-/-}$ Treg cells to modulate Th1-mediated inflammation during infection.[19] STAT3-deficient Treg cells have reduced expression of CCR6, an important chemokine receptor directing recruitment to sites of Th17-mediated inflammation.[43] STAT3-deficient Treg cells also showed reduced expression of other genes related to the suppressive function of Treg cells, including *Il10*, *Ebi3* and *Gzmb*,[43] these genes are also down-regulated in IRF4$^{-/-}$ Treg cells.[40] The inability of T-bet$^{-/-}$ and STAT3$^{-/-}$ Treg cells to control highly polarized responses is associated with impaired migratory potential and reduced IL-10 production. Indeed the elevated suppressive functions seen in Th1-conditioned Treg cells (activated via STAT1), in IL-10-exposed Treg cells (activated via STAT3) and in IRF4$^+$ Treg cells[42] can all be attributed to increased IL-10 production, rather than to distinct subset-specific suppressive mechanisms. Although

IL-10 can suppress both Th1 and Th17 responses, Th17 cells are particularly sensitive to IL-10-mediated suppression (due to increased expression of the IL-10ra[44]), which may explain why reduced IL-10 production by STAT3-deficient Treg cells favours outgrowth of Th17 responses.[43]

Effector tuned Treg cells: specialists or chameleons?

Until recently it was unclear whether gain of effector cytokine production in Treg cells represented a committed functional change or whether Treg cells could switch back and forth between co-expression of effector-associated transcription factors as dictated by prevailing conditions. Recent fate mapping studies tracing the expression of Foxp3, Gata3 and T-bet in Treg cells show dynamic changes in the co-expression of transcription factors. In the steady-state around 30% of Treg cells express Gata3 and around 10% express T-bet. Interestingly, a small subset of Treg cells co-express Foxp3, T-bet and Gata3.[45] Furthermore, these patterns of expression are highly flexible. T-bet$^+$ Gata3$^-$ Treg cells can be induced to lose expression of T-bet and gain expression of Gata3 and vice versa.[45] Hence T-bet$^+$ and Gata3$^+$ Treg cells are not stable subsets. Instead, Treg cells adapting to a strong Th1 response with acquisition of T-bet expression could return to basal levels of T-bet expression as the response wanes and retain the potential to 're-tune' to subsequent inflammatory episodes.

Calibration of the Treg response to the degree of inflammation

Cytokine production by non-T cells and stromal cells during inflammation or tissue damage can also influence Treg function. For example, the 'alarmin' IL-33 which is released during necrotic cell death regulates Treg dynamics *in vivo* and this pathway is of particular importance at inflammatory sites.[46–48] A subset of highly activated Treg cells express ST2 (the IL-33 receptor) and respond to IL-33 with increased proliferation, activation and elevated Gata3 expression. Interleukin-33 also increased expression of molecules involved in tissue homing (CD103, αV integrin, OX40, CD44) and suppressive function (CTLA-4, PD-1, CD39, CD73, LAG3).[48,49] These results suggest that IL-33 promotes expansion of Treg cells capable of homing to sites of inflammation and displaying increased suppressive capacity. Indeed, ST2$^+$ Treg cells may be particularly well adapted to survival in tissues as they are enriched in fat, muscle and skin compared with secondary lymphoid organs.[33,50,51] Notably, the Gata3-promoting activity of IL-33 is suppressed by IL-23, demonstrating a further way in which IL-23 can act to destabilize the Treg phenotype.[48] ST2$^{-/-}$ Treg cells also fail to protect against T-cell-induced colitis, an inadequacy also seen in Gata3$^{-/-}$ Treg cells.[29,30] As IL-33 is constitutively expressed in epithelial and endothelial cells, this pathway represents a novel means whereby cells out-with the immune system can act to promote Treg stability and function during inflammation and one which might be of particular importance at barrier sites. ST2 expression is also an essential feature of Treg cells found in visceral adipose tissue. This ST2 expression was dependent on IRF4, highlighting a further key influence of Treg function, and basic leucine zipper transcription factor ATF-like (BATF). Interestingly, both of these transcriptional regulators are responsive to IL-33, allowing a positive feedback loop for the propagation of highly functional Treg cells.[52]

Plasticity, stability or heterogeneity?

The origin of cells co-expressing Foxp3 and effector cytokines has been controversial. Do all Treg cells possess equivalent potential for functional plasticity or is it restricted to Treg subsets that are either inherently unstable or exist with a propensity to acquire particular effector characteristics?

Responsiveness to switch factor cytokines requires expression of the appropriate cytokine receptor and this can therefore predict the capacity of Treg cells for inflammatory cytokine production. The proportion of Treg cells induced to produce IL-17 *in vitro* never equals the level of induction seen following stimulation of naive T cells in the presence of TGF-β and IL-6.[7,11] The differential sensitivity of naive conventional T cells and Treg cells to IL-6 reflects their differential patterns of IL-6Ra expression (only around 30% of Treg cells express IL-6Ra). Highly activated Treg cells are IL-6Ra-negative, fail to phosphorylate STAT3 in response to IL-6[38] and cannot be induced to produce IL-17.[9] Therefore reduced sensitivity to IL-6 in Treg cells at inflammatory sites can serve as a potent means of stabilizing their suppressive function. Underlining this, Treg cells isolated from the inflamed CNS during EAE are insensitive to IL-6 because they do not express either IL-6Ra, or the signal transducing sub-unit gp130. In contrast, splenic Treg cells from the same mice do express IL-6 receptors and are capable of IL-6-driven IL-17 production.[38] In a parallel situation, IL-1R1 expression identifies a subset of CCR6$^+$ ROR-γT$^+$ human Treg cells that can produce IL-17 in response to IL-1β.[53] Acquisition of Th1-like cytokine production in murine Treg cells requires intact IFN-γ-signalling. Exposure to IFN-γ also induces IL-12RB2 expression, allowing IL-12 to further promote T-bet expression.[27] Hence, patterns of cytokine receptor expression can identify populations of Treg cells with a predisposition to effector-cytokine production.

Historical fate-mapping of gene expression formally demonstrated the capacity for Treg cells to lose Foxp3 expression. Transfer of sorted GFP$^+$ Treg cells from Fox-

p3-GFP knock-in mice provided the first direct evidence that up to 50% of Treg cells can lose Foxp3 expression after transfer to the lymphopenic environment of Rag2$^{-/-}$ mice.[54] Furthermore cells that had lost Foxp3 expression subsequently gained a capacity for production of IL-2 and IFN-γ and showed pathogenic potential upon secondary transfer (see below).[54] To quantify loss of Foxp3 expression in immunocompetent hosts, it was necessary to develop dual reporter mice allowing simultaneous assessment of current and historical expression of Foxp3 to be visualized in live cells.[55] These studies estimated that around 10% of cells that, at some time had expressed Foxp3, now did not. These so-called 'ex-Treg' cells displayed an activated effector-memory phenotype and a substantial proportion of them could produce either IFN-γ or IL-17.[55] Furthermore, an elevated frequency of ex-Treg cells in the pancreas of diabetic NOD mice, suggested that these cells may function in a pro-inflammatory capacity at sites of autoimmune inflammation.[55] Auto-reactive ex-Treg cells have the potential to contribute to inflammation in the CNS as transferring ex-Treg cells from autoantigen-immunized mice can induce EAE in Rag$^{-/-}$ mice (see below).[56] Similarly, transfer of *in vitro*-expanded BDC2.5 ex-Treg cells (which express a TCR that recognizes a pancreatic islet autoantigen) can induce diabetes in NOD Rag2$^{-/-}$ recipients.[55] Collectively these studies illustrate plasticity in Treg cells, which can give rise to ex-Treg cells capable of driving autoimmune inflammation.

Opposing results suggest that fully committed Treg cells are functionally stable. Using a pulse-labelling approach in which cells expressing Foxp3 were induced to permanently express YFP following exposure to Tamoxifen, it was found that under homeostatic, lymphopenic and inflammatory conditions there was very little evidence for loss of Foxp3 expression, or gain of effector function.[57] A possible explanation for the different observations is that, in the original dual reporter mouse, transient or low-level expression of Foxp3 either during Th17 differentiation[10] or in peripherally induced Treg cells may have led to an accumulation of cells with an ex-Treg phenotype over time. The pulse-labelling approach avoids the accumulation of these cells in the 'ex-Treg' pool.

A third school of thought argues that phenotypic changes reflect Treg heterogeneity, suggesting that the majority of Treg cells are functionally stable but that a minor subpopulation has the capacity for functional change.[58,59] This could account for the conflicting results of those studies advocating plasticity versus stability. As mentioned previously, transfer of Foxp3^{+} Treg cells to a lymphopenic environment results in loss of Foxp3 expression in a proportion of Treg cells. However, if Treg cells are sorted into CD25^{+} and CD25^{-} fractions before transfer, the CD25^{+} fraction retains stable Foxp3 expression, whereas Foxp3 is readily lost from the CD25^{-} fraction.[59] Notably, loss of Foxp3 was not permanent as activation of ex-Treg cells *in vivo* or *in vitro* could re-induce Foxp3 expression in those studies. A highly sensitive reporter system (in which membrane-bound human CD2 is expressed on the surface of T cells expressing Foxp3) demonstrated that, during activation, a small proportion of murine T cells transiently up-regulate Foxp3 expression.[58] Hence, the 'ex-Treg' population identified in previous studies is not composed solely of Treg cells that have lost Foxp3 expression but could well be accounted for in large part by cells that have abortively or transiently expressed Foxp3 during activation.

This transient expression of Foxp3 during activation of non-Treg cell populations has been observed by others[60] and highlights the need for additional factors to drive regulatory function. Stable Foxp3 expression is associated with pronounced CpG demethylation that occurs in the Treg-specific demethylation region (TSDR) within the Foxp3 gene.[61] Interestingly, further epigenetic studies of natural Treg cells have revealed Treg-specific CpG demethylation profiles in other genes. These epigenetic profiles occurred independently of Foxp3 expression, but had complementary roles in establishing Treg function.[62,63]

The pro-inflammatory potential of Treg cells: the bark and the bite

However ex-Treg cells arise, their pro-inflammatory potential makes them highly relevant to the development of Treg-based therapies. Autoantigen-reactive Treg cells provide the best preventive and the only curative therapy for experimental autoimmune disease.[64,65] For conditions where the autoantigen is known, *in vitro* expansion of antigen-reactive Treg cells for therapeutic application is achievable.[66,67] However, it is not hard to imagine the potentially disastrous outcome of transfusing large numbers of autoreactive Treg cells with the capacity to respond to inflammation by losing Foxp3 expression and gaining effector function. We should take a moment to ask how well these fears are substantiated by current evidence.

The enrichment of autoreactive TCR within Treg cells and the prevalence of ex-Treg cells at sites of autoimmune inflammation prompted Zhou *et al.*[55] to propose that autoimmune disease may arise, not from the activation of conventional autoreactive T cells, but from the repurposing of autoreactive Treg cells. Although this may be possible in certain circumstances, the scurfy-like phenotype that develops following diphtheria toxin-mediated deletion of Treg cells[68,69] proves that redirection of Treg cells to effector function is not required to initiate autoimmune disease. The development of exacerbated autoimmune diseases following Treg depletion,[39,70] of autoimmune inflammation in immunodeficient mice fol-

lowing transfer of naive T cells[71,72] and of spontaneous disease in TCR transgenic mice lacking Treg cells,[73] shows that autoimmune disease can develop in the absence of Treg cells. Similarly, EAE can be successfully induced in Treg-depleted mice, indicating there is no requirement for effector cytokine production by CNS autoantigen-reactive Treg cells to initiate disease.[70]

Although Treg cells are not required for autoimmune disease, it remains unclear to what degree ex-Treg cells can contribute to ongoing inflammation. Deletion of Foxp3 in mature Treg cells leads to loss of suppressor function and the resultant Foxp3^{-} Treg cells were unable to prevent the development of severe autoimmune disease when co-transferred with naive T cells into lymphopenic hosts.[74] Furthermore ex-Treg cells were predominant among the T-cell infiltrate in the heavily inflamed skin, lung and intestines.[74] Although this evidence suggests that ex-Treg cells can drive pathology, loss of Foxp3 was forced and irreversible rather than naturally occurring and dynamic. Furthermore, there was no demonstration of ex-Treg cells driving inflammation in the absence of co-transferred conventional T cells.

There is direct evidence for ex-Treg cells driving autoimmune disease but their pathogenic potential is seen only in very particular circumstances, most commonly, after serial transfer into lymphopenic hosts (see Fig. 1). After transfer of Treg cells into Rag2$^{-/-}$ mice, a proportion of cells lose Foxp3 expression. Upon secondary

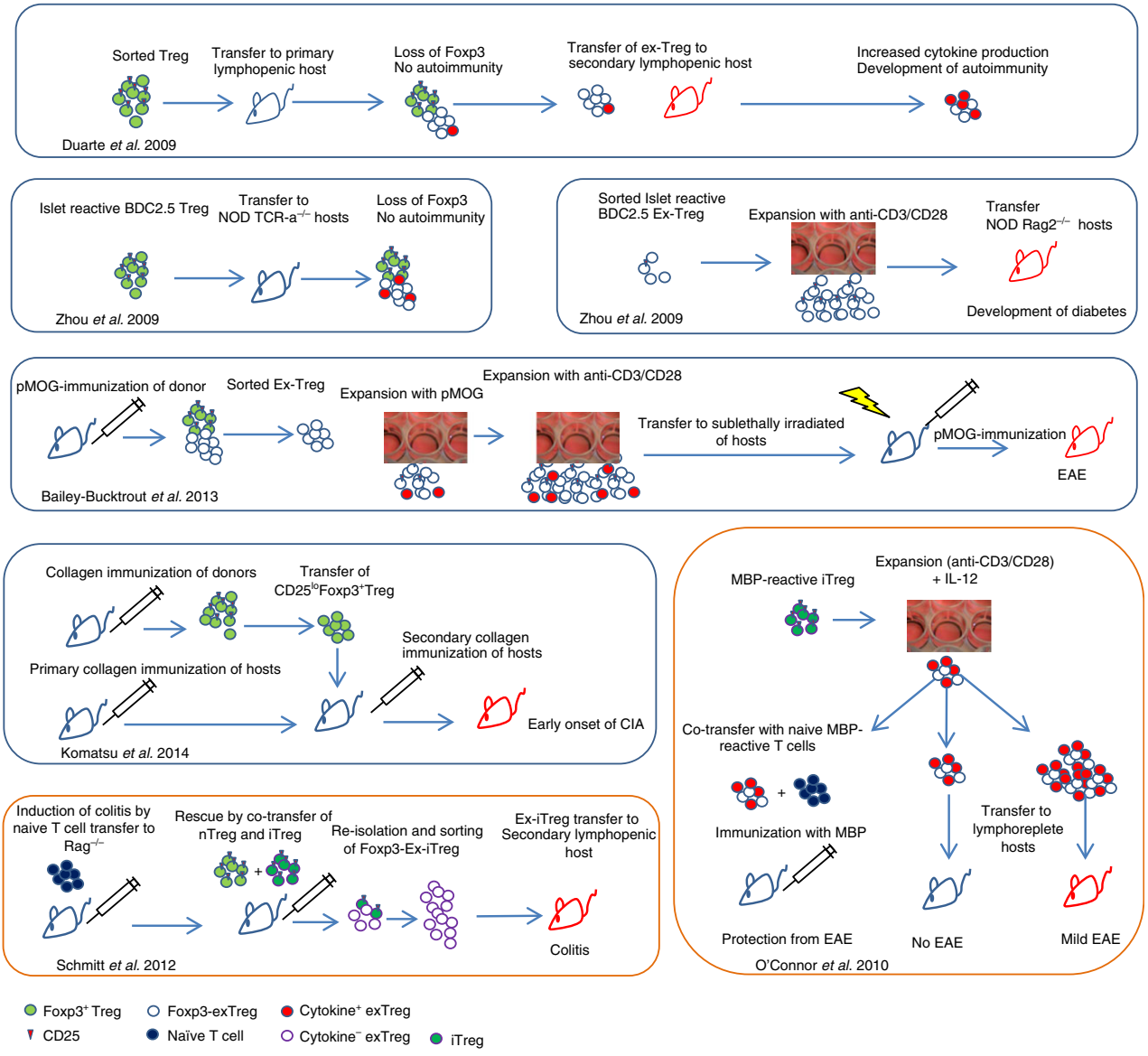

Figure 1. Schematics illustrating the experimental protocols under which Foxp3^{+} regulatory T cells have been shown to acquire a capacity for pro-inflammatory cytokine production and the potential to drive autoimmune pathology.

transfer into TCR-$\beta^{-/-}$ mice Foxp3^{-} cells gained effector function and induced autoimmune inflammation.[54] Importantly, inflammatory cytokine production increased only after transfer of the ex-Treg to the second lymphopenic host. The presence of stable Treg cells (which retained their suppressive capacity) in the primary transfer is therefore sufficient to prevent gain of effector function by ex-Treg cells, even after loss of Foxp3. This is also true in models using autoreactive Treg cells from transgenic mice. After transfer of BDC2.5 Treg cells to TCRa$^{-/-}$ hosts, around a third of these cells lost Foxp3 expression and gained a capacity for IFN-γ production. Despite this, recipient mice did not develop diabetes.[55] Sorting of ex-Treg cells followed by their *in vitro* expansion before transfer to NOD Rag2$^{-/-}$ mice was required to induce diabetes.[55] Similarly, *in vitro* expansion of ex-Treg cells from CNS autoantigen-immunized mice, generated a population of ex-Treg cells capable of inducing EAE after transfer to sub-lethally irradiated hosts.[56] Hence there is no evidence for ex-Treg cells initiating disease on a primary transfer without immunization. Most often, *in vitro*[55,56] or *in vivo*[54] activation in the complete absence of other T cells is required for ex-Treg cells to gain full effector function. Although they may sound unusual, such conditions are directly relevant to the potential therapeutic application of Treg cells, particularly for treatment of graft versus host disease, where *in vitro* expanded Treg cells are transferred following bone marrow transplantation (reviewed in ref. 75).

The potential of Treg, or ex-Treg, cells to contribute to ongoing inflammation has not yet been directly ascertained. This would require selective deletion of the capacity for effector cytokine production in Treg cells. Using the same techniques employed to determine the role of Teff-associated transcription factors, but deleting only effector cytokines, it would be possible to allow unimpaired regulation of bona-fide Teff responses while eliminating effector function in Treg and ex-Treg cells. Of course, it is possible (and perhaps likely) that removing effector function in a minor population of cytokine-producing cells would have a limited effect on the clinical course of disease, especially in EAE where global deletion of either IFN-γ[76] or IL-17[77] does not prevent development of disease. However, a demonstration of reduced pathology in the absence of effector function specifically in Treg cells would provide the most convincing demonstration that Treg cells can contribute to, as well as suppress, inflammation. The best evidence to date that cells which have expressed Foxp3 can contribute to inflammation comes from adoptive transfer of CD25^{lo} Foxp3^{+} (unstable) Treg cells from collagen-immunized DBA-1 mice into previously immunized DBA-1 hosts before secondary immunization.[13] This protocol resulted in an earlier onset of arthritis in recipient mice. Notably transfer of CD25^{hi} Foxp3^{+} Treg cells did not accelerate pathology, this supports the concept of Treg heterogeneity and indicates that CD25^{hi} cells are stable, fully committed Treg cells.

Stability of induced Treg cells

T cells induced to express Foxp3 *in vitro* by stimulation in the presence of TGF-β and IL-2 (called inducible, or iTreg cells)[6] display only partial demethylation of the Foxp3 TSDR and readily lose expression of Foxp3 upon *in vitro* re-stimulation.[61] However, despite loss of Foxp3, ex-iTreg cells retain suppressive capacity *in vitro* and are able to suppress development of EAE and colitis *in vivo*.[78,79] The iTreg cells cannot be induced to produce IL-17 by IL-6 (due to their lack of IL-6ra expression[80]) but can readily be induced to produce IFN-γ by IL-12.[78,79] iTreg cells that have been induced to lose Foxp3 expression can induce colitis. However, this again required serial transfer into secondary lymphopenic hosts.[81] When transferred to lymphoreplete hosts, even large numbers of IFN-γ^{+} myelin-reactive ex-iTreg cells failed to induce EAE, emphasizing the importance of the environment in setting the threshold for pathogenic potential.[79] Inducible Treg cells can also produce granulocyte–macrophage colony-stimulating factor (GM-CSF) upon secondary stimulation,[82] which is of relevance given the central role of this cytokine in EAE.[83,84] However, production of GM-CSF by iTreg cells is susceptible to inhibition by IL-6, IL-27, IL-2 and TGF-β. Hence, although iTreg cells are prone to lose Foxp3 expression, they retain some suppressive activity and cross-regulation by cytokines produced by conventional T cells and antigen-presenting cells inhibits their pro-inflammatory potential in immunocompetent hosts.[82]

Factors promoting Treg stability

Interleukin-2 maintains Foxp3 expression[85] and prevents effector function in Treg cells.[30,54,56] Treatment with IL-2 : anti-IL-2 complexes increases Treg numbers, elevates Gata3 expression and prevents Th17-deviation.[30] During *Toxoplasma* infection, IL-2 : anti-IL-2 complexes similarly reduced T-bet expression in Treg cells, prevented collapse of the Treg population and increased survival.[20] Beneficial effects have also been reported in EAE, wherein IL-2-complexes reduced the frequency of ex-Treg cells and improved clinical outcome.[56] Hence IL-2 can prevent the development of both Th1 and Th17 characteristics in Treg cells and may provide a means to promote Treg stability during inflammation or following therapeutic delivery of Treg cells.

Increased Treg stability could also be achieved by reducing the influence of switch factor cytokines such as IFN-γ and IL-6. Suppressor of cytokine signalling 1 (SOCS-1) decreases phosphorylation of STAT1 and

STAT3, which drive Treg cells towards Th1 and Th17 differentiation, respectively.[86] SOCS-1-deficient Treg cells rapidly lose Foxp3 expression and readily produce effector cytokines in lymphopenic environments.[86] SOCS-1 is highly expressed in Treg cells,[87] and is required for their suppressive function.[88] Hence inhibition of Janus kinase (JAK)-STAT-signalling may also provide a therapeutic means to stabilize Treg suppressive function.

The Foxp3 TSDR is also known as conserved non-coding sequence 2 (CNS2). As mentioned above, this intronic *cis*-element plays a key role in maintaining Foxp3 expression during active Treg proliferation[89] and CNS2-deficient mice develop lymphoproliferative disease with multi-organ immune infiltration.[90] CNS2 appears able to interact with the Foxp3 promoter via a short-distance loop, promoted through NFAT binding, providing a means by which TCR-stimulation can drive CNS2-mediated Foxp3-stabilization.[90] A contemporaneous study of CNS2 suggested that, at least part of the functional consequence of the CNS2-Foxp3 promoter interaction was to deliver STAT5 to the promoter, thereby facilitating the action of IL-2 on Foxp3-stability. Moreover, in the absence of CNS2, the loss of Foxp3 upon exposure to pro-inflammatory cytokines was enhanced.[91] This leads to a model in which pro-inflammatory STATs (e.g. STAT3 and STAT6) can deliver DNA methyltransferases that would extinguish Foxp3 gene transcription, an outcome that is prevented by the concerted actions of TCR stimulation, IL-2/STAT5 signalling and CNS2 function. Importantly, as these studies showed, the two net effects in Treg cells are maintained Foxp3 expression and resistance to gain of effector cytokine production.[90,91]

Treg-associated cytokines can also promote Treg stability. Interleukin-10 increases the ability of Treg cells to control Th17 responses [43] and prevents induction of T-bet and IFN-γ,[92] while TGF-β stabilizes the phenotype of human Treg cells.[15,59] Hence IL-2, IL-10 and TGF-β can all act to strengthen the Treg transcriptional programme.

Another means of promoting Treg stability is to target those factors that would erode it. Treatment with anti-IL-12 and anti-IFN-γ enhance the suppressive capacity of human Th1-like Treg cells[22] and the IL-1 receptor antagonist reduces IL-17 production induced by IL-1β.[14] The balance of pro- and anti-inflammatory signals can therefore profoundly influence both suppressive capacity and cytokine production.

In conventional T cells, the E2-ubiquitin conjugating enzyme Ubc13 (Ubc13) regulates activation of the inhibitor of nuclear factor-κB kinase IKK and nuclear factor-κB during TCR-mediated activation.[78] Treg-specific deficiency in Ubc13 does not reduce Treg numbers, but does lead to spontaneous autoimmune disease. Ubc13-deficient Treg cells lack suppressive function and this is associated with decreased expression of both IL-10 and SOCS-1.[79] The combined influences of TCR stimulation,[52,53] IL-2 deprivation[52,53] and cytokine exposure[14,30,54] in a lymphopenic environment[51–53] seem to be required to promote Treg conversion to a full effector phenotype. This level of control is probably appropriate and would allow for gain of inflammatory function only in the absence of conventional T cells (which are better-suited to take on this role), and only in the presence of switch factors such as IL-6, IL-23, IL-1β and IL-12 (all indicative of inflammation, requiring activation of an adaptive immune response). Hence gain of effector function in these extreme settings could be a case of needs must when the devil drives.

Is Treg stability always desirable?

Suppression of anti-tumour immune responses by Treg cells can preserve a microenvironment favouring tumour growth. Interfering with Treg function provides a therapeutic opportunity to promote anti-tumour responses. Taking advantage of Treg plasticity might allow local manipulation of Treg function to achieve this end without the need for Treg depletion.

The transcriptional co-repressor Eos (Ikzf4) forms a complex with Foxp3, suppressing production of IL-17 and maintaining Treg cells in suppressive mode.[93] Upon TCR stimulation, a subset of Treg cells can lose expression of Eos and begin to produce IL-17. Down-regulation of Eos is induced by IL-6 and can be blocked by the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO), which is produced in large amounts by plasmacytoid dendritic cells in tumour-draining lymph nodes. Inhibition of IDO increases IL-17-production by Treg cells and promotes effective anti-tumour immunity.[94,95] Similarly, overcoming Treg-mediated suppression by local delivery of IL-12 (in combination with CTLA-4 blockade) allows eradication of tumours in a murine model of glioblastoma.[96] In colon cancer the frequency of ROR-γT$^+$ IL-17$^+$ Treg cells among peripheral blood mononuclear cells increases with the transition from stage II to stage III disease, indicating that this readout could be a useful biomarker of disease progression.[97] Genetically polyp-prone mice also have an elevated frequency of ROR-γT Treg cells and deletion of ROR-γT specifically within Treg cells provides increased immunosurveillance and decreased polyposis, demonstrating its relevance to disease.[97] These studies show that local modulation of Treg function (by either provision or inhibition of switch factor cytokines) provides a means to effectively modulate immune responses. Hence, when the clinical imperative is to alleviate the Treg-mediated suppression of beneficial anti-tumour immune responses, the capacity of IL-12 and IL-6 to destabilize Treg cells and promote plasticity could be manipulated therapeutically.

Regulatory, T regulatory

Treg cells were first identified as a subset of T cells with the capacity to prevent the development of spontaneous autoimmune disease. In accordance with this remit, non-suppressive functions have been viewed in part as aberrant, deviant behaviours, despite the frequency with which they are reported. Thankfully however, these enigmatic cells were rightly named 'regulatory T cells'. To live up to the name, their ability to adjust the mechanisms of the immune system must work both ways. Although the natural consequence of loss of suppressive function and gain of effector function in Treg cells is uncertain, a great deal of information has accrued on where and when it is likely to happen and which factors favour or inhibit conversion. Armed with this knowledge we are better positioned to design Treg-based therapies. For example, including strategies to promote functional stability after Treg transfer (via provision of IL-2), or locally altering Treg function to promote anti-tumour immunity. There is also increasing recognition of the roles of Treg cells in non-lymphoid organs to impact upon obesity[50] and wound healing[51,98] as well as their more traditionally recognized suppressive roles. The recent discovery that a subset of TIGIT$^+$ Treg cells suppress the development of Th1 and Th17 responses while favouring Th2 polarization goes further and demonstrates that Treg cells can shape development of the adaptive immune response from the outset.[99] As appreciation of their diverse functions grows, our understanding of Treg cells becomes less one-dimensional and the capacity to modulate immunity by targeting Treg function only looks set to increase.

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Disclosures

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