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Phenotypic and functional specialization of FOXP3+ regulatory T cells

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Abstract

Forkhead box P3 (FOXP3)$^+$ regulatory T (T_{Reg}) cells prevent autoimmune disease, maintain immune homeostasis and modulate immune responses during infection. To accomplish these tasks, T_{Reg} cell activity is precisely controlled, and this requires T_{Reg} cells to alter their migratory, functional and homeostatic properties in response specific cues in the immune environment. We review progress in understanding the diversity of T_{Reg} cells, T_{Reg} cell function in different anatomical and inflammatory settings, and the influence of the immune environment on T_{Reg} cell activity. We also consider how these factors impact immune-mediated disease in the contexts of infection, autoimmunity, cancer and transplantation.

Forkhead box P3 (FOXP3)$^+$ regulatory T (T_{Reg}) cells function to maintain immune tolerance and prevent inflammatory diseases[1]. This is best exemplified by the severe systemic autoimmunity and lymphoproliferative disease observed in T_{Reg} cell-deficient mice and humans carrying non-functional or hypomorphic alleles of the *FOXP3* gene. The impaired function and/or homeostasis of T_{Reg} cells have also been implicated in development of several common autoimmune and inflammatory diseases, including type-1 diabetes, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus[2–5]. In addition to preventing autoimmunity, T_{Reg} cells can also regulate immunity to infections of viral, bacterial or parasitic origin as well as restrain anti-tumor and anti-transplant immune responses[6, 7]. Thus, T_{Reg} cells must 'walk the line' by allowing protective anti-tumor and anti-pathogen immunity while preventing autoimmune disease by restraining aberrant responses to self or to innocuous antigens.

To function properly, T_{Reg} cells must modulate the activities of a wide variety of cellular components of both the innate and adaptive immune systems, and this depends on their ability to come into physical proximity with their targets by migrating to specific tissues and microenvironments. Additionally, it is critical that T_{Reg} cells elaborate an appropriate immunomodulatory mechanism that will effectively inhibit the targeted cell population. Recently, it has become clear that T_{Reg} cells can be divided into several distinct subsets displaying unique functional and homeostatic properties that work in concert to maintain normal immune homeostasis[8]. In this Review, we will summarize recent advances in our understanding of the relationship between T_{Reg} cell trafficking and function in lymphoid and non-lymphoid tissues, T_{Reg} cell specialization during different types of immune responses, and the impact of the cytokine milieu on the phenotype, function and stability of T_{Reg} cells.

Widespread distribution of T_{Reg} cells

T_{Reg} cells localize to lymphoid and non-lymphoid sites

The cells and tissues of the immune system are anatomically organized to facilitate cellular interactions required for the development, activation, function and regulation of diverse leukocyte populations [9]. The organization of the immune system is the result of tissue- and microenvironment-specific lymphocyte homing, which in turn is mediated by lymphocyte expression of surface adhesion and chemoattractant receptors. Although T_{Reg} cells were initially identified in the secondary lymphoid tissues of mice and peripheral blood of humans, they express a dizzying array of adhesion molecules and chemoattractant receptors expected to target them to both lymphoid and non-lymphoid sites (Table 1). Indeed, in addition to their constitutive presence in secondary lymphoid tissues, T_{Reg} cells can be found in most non-lymphoid tissues, even in the absence of any overt inflammation[10]. Additionally, T_{Reg} cells can be found in abundance within tumors, where they are thought to help blunt effective tumor clearance[7]. In recent years, genetic studies have revealed the importance of several homing receptors for the appropriate tissue distribution and function of T_{Reg} cells. For instance, expression of the αE integrin chain (also known as CD103) and the chemokine receptor CC-chemokine receptor 4 (CCR4), as well as the ability to generate carbohydrate ligands for P- and E-selectin by the action of the α(1,4)-fucosyltransferase VII enzyme are all important for the migration and/or retention of T_{Reg} cells within the skin. Accordingly, deletion of any of these molecules on T_{Reg} cells results in development of skin-specific autoimmunity and altered pathogen clearance during cutaneous infection[10–13]. Similarly, loss of CCR7 blocks T_{Reg} cell migration to the lymph nodes, and inhibits T_{Reg} cell function in an experimental model of colitis[14].

In addition to their constitutive recirculation, T_{Reg} cell recruitment to non-lymphoid tissues is substantially enhanced during inflammation. However, the contributions of individual homing receptors to this ‘inflammation-induced’ T_{Reg} cell migration vary considerably depending on the tissue involved and the type of inflammatory response. For instance, interleukin-17 (IL-17) produced during T helper 17 (T_H17) cell-mediated inflammation promotes epithelial cell expression of the CCR6 ligand, CC-chemokine 20 (CCL20), and CCR6 is essential for the optimal recruitment of T_{Reg} cells to sites of T_H17-mediated inflammation during experimental autoimmune encephalomyelitis (EAE)[15]. Similarly, interferon-γ (IFNγ) induces expression of the CXC-chemokine receptor 3 (CXCR3) ligands CXC-chemokine ligand 9 (CXCL9), CXCL10 and CXCL11 and the subsequent recruitment of $CXCR3^+$ T_{Reg} cells to the liver during conA-induced hepatitis[16]. Additionally, CXCR3 may influence the microenvironmental positioning of T_{Reg} cells in the central nervous system during EAE[17]. In this fashion, recruitment of $CCR6^+$ and $CXCR3^+$ T_{Reg} cells may act downstream of the key effector cytokines IL-17 and IFNγ in a feedback loop to limit T_H17- and T_H1-induced inflammatory responses.

In addition to CCR6 and CXCR3, many other homing receptors have been implicated in inflammatory recruitment of T_{Reg} cells in different immunological settings, including CCR1, CCR2, CCR4, CCR5, CCR8, CCR9, CXCR4, CXCR5, CXCR6, α4β1 integrin (also known as VLA4), $\alpha_E\beta7$ integrin, α4β7 integrin and the P- and E-selectin ligands. Indeed, T_{Reg} cells most likely use a combination of homing molecules that can function redundantly to control their migration during inflammatory responses. For example, in an islet allograft model, T_{Reg} cells used CCR2, CCR5, CCR4 and P- and E-selectin ligands to migrate to the transplant site, whereas CCR2, CCR5 and CCR7 were required for trafficking from the inflamed allograft to the draining lymph node[18]. Interestingly, this sequential migration of T_{Reg} cells from the graft site to the draining lymph node was required for prevention of graft rejection, highlighting the complex and dynamic nature of T_{Reg} cell migration during inflammation. Thus, therapeutically targeting T_{Reg} cell migration in the contexts of

autoimmunity, transplantation, chronic infection and cancer remains a daunting task that will require significantly more study in both animal models and patient populations.

Differences in T_{Reg} cells in lymphoid vs. non-lymphoid tissues

Although T_{Reg} cells can be found throughout the body, there is substantial phenotypic and functional variation between T_{Reg} cells in lymphoid and non-lymphoid tissues. Developing T_{Reg} cells in the thymus are a relatively homogenous population of $CD25^{hi}CD62L^{+}CCR7^{+}$ cells that resemble conventional naive T cells and preferentially migrate to secondary lymphoid tissues. However, upon entering the periphery, a subset of T_{Reg} cells rapidly acquires phenotypic features of effector or memory T cells, becoming $CD44^{hi}$ and upregulating expression of homing receptors that allow them to access non-lymphoid sites[19, 20]. Moreover, similar to conventional naive T cells and effector and memory T cells, $CD44^{lo}$ and $CD44^{hi}$ T_{Reg} cell subsets have distinct homeostatic characteristics, with $CD44^{hi}$ T_{Reg} cells proliferating at a significantly higher rate in the steady-state[21]. This suggests that there is a 'division of labor' between distinct T_{Reg} cell subsets specialized for functioning in lymphoid or in non-lymphoid tissues. In addition, the phenotype of the $CD44^{hi}$ effector/memory-like T_{Reg} cell population indicates that it arises as a result of T_{Reg} cell activation, presumably due to recognition of self-antigens in secondary lymphoid tissues (Box 1).

Although the functional mechanisms used by T_{Reg} cells are complex and still incompletely understood, there is increasing evidence that T_{Reg} cells use different mechanisms to regulate immune responses in lymphoid and in non-lymphoid tissues. This concept is best exemplified by the distinct phenotypes in mice lacking expression of either IL-10 or cytotoxic T lymphocyte antigen 4 (CTLA4) selectively in T_{Reg} cells (Figure 1). IL-10 is a cytokine that is produced by $CD44^{hi}$ T_{Reg} cells and can both directly and indirectly inhibit effector T cell responses during infection, autoimmunity and cancer[22–24]. Although it is also expressed by other leukocyte populations, deletion of IL-10 selectively in T_{Reg} cells resulted in the development of spontaneous colitis, and exaggerated immune responses at other environmental interfaces such as the skin and lungs[25]. However, these animals did not develop the systemic autoimmunity and dysregulated T cell activation profiles characteristic of mice with a complete loss of T_{Reg} cell function. By contrast, loss of CTLA4 expression in T_{Reg} cells resulted in severe lymphoproliferative disease characterized by massive lymphadenopathy and splenomegaly associated with accumulation of $CD4^{+}CD44^{hi}$ effector T cells, spontaneous multi-organ autoimmunity, and early death[26].

Immunoregulation by T_{Reg} cell-expressed CTLA4 is due, at least in part, to the ability of CTLA4 to render dendritic cells (DCs) in lymphoid tissues less immunostimulatory by downregulating their surface expression of the co-stimulatory ligands CD80 and CD86[26]. Additionally, CTLA4 ligation of CD80 and CD86 on DCs can induce expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO)[27, 28]. Together, these activities can raise the threshold required for T cell activation, thereby preventing the priming of autoreactive T cells. Indeed, time-lapse imaging studies have revealed that T_{Reg} cells form long-lasting, stable contacts with DCs, and this has led to the hypothesis that a primary mode of T_{Reg} cell-mediated suppression within lymphoid tissues may be through inhibition of DC activation and/or function[29, 30]. T_{Reg} cells can also induce perforin-dependent cytolysis of DCs in tumor draining lymph nodes[31]. Thus, T_{Reg} cells use multiple mechanisms to limit DC activity in secondary lymphoid tissues, thereby quelling effector T cell activation and promoting functional tolerance. Accordingly, acute depletion of T_{Reg} cells in mice leads to the rapid development of systemic autoimmunity, associated with increases in both the number and activation state of DCs in lymphoid tissues[32]. The importance of T_{Reg} cell function in secondary lymphoid tissues extends beyond their integral role in preventing autoimmunity. For instance, depletion of T_{Reg} cells following intravaginal infection with herpes simplex virus 2 (HSV2) accentuated T cell priming and

proliferation in the draining lymph node, but prevented effector T cell mobilization from the lymph node to the vaginal epithelium, resulting in uncontrolled viral replication and death. These results highlight a previously unappreciated function of T_{Reg} cells in down-modulating immune responses in the lymphoid tissues to allow for efficient effector T cell migration to sites of infection[33].

The spontaneous phenotypes of mice lacking either IL-10 or CTLA-4 in T_{Reg} cells demonstrate that these molecules are essential for proper T_{Reg} cell function *in vivo*. However, numerous other mechanisms have been implicated in T_{Reg} cell function in both lymphoid and non-lymphoid tissues in various experimental settings[34]. These include production of additional immunosuppressive cytokines such as transforming growth factorβ (TGFβ) and IL-35, along with metabolic inhibition of effector T cells through adenosine or cyclic AMP. Thus, T_{Reg} cells use a variety of immunosuppressive mechanisms to modulate both the initiation of the immune response in secondary lymphoid tissues, and the progression and termination of inflammatory responses at non-lymphoid sites, and the suppressive module used by T_{Reg} cells appears to be a function of both the tissue site and character of the inflammatory response. Indeed, it has been difficult to determine the importance of specific immunoregulatory mechanisms used by T_{Reg} cells, indicating that although T_{Reg} cells are clearly essential for establishing and maintaining tolerance, substantial functional redundancy may exist in the means they employ to do so. Further unraveling the complex relationship between T_{Reg} cell localization and function will likely yield important new insights into the functional diversity of T_{Reg} cells, and in understanding how specific immunomodulatory functions are delivered to different tissues during the course of the immune response.

T_{Reg} cell control of distinct immune responses

Functional specialization of T_{Reg} cells

$CD4^+$ effector T cells can adopt one of several functional fates and elaborate distinct effector mechanisms depending on the cytokines present during their initial activation[35]. Production of these polarizing cytokines is dictated by the type of pathogen encountered, such that TGFβ and IL-6 direct the development of IL-17-producing T_H17 cells during extracellular bacterial or fungal infection, IFNγ and IL-12 drive the differentiation of IFNγ-producing T_H1 cells that help combat intracellular pathogens, and IL-4 induces IL-4-producing T_H2 cells during infection with large mucosal parasites[36–38]. The functional specialization of these various $CD4^+$ T cell subsets is due to the differential expression of 'master' transcription factors, namely retinoic acid receptor-related orphan receptor-γt (RORγt), T-bet and GATA-binding protein 3 (GATA3), which turn on distinct programs of gene expression controlling T cell function and migration[39–41]. However, each of these responses is pro-inflammatory and potentially harmful to host tissues. Accordingly, aberrant T_H1, T_H2 and T_H17 responses can all contribute to immunopathology in the contexts of infection, autoimmunity, allergy and other inflammatory conditions[42, 43]. Therefore, these distinct immune responses must be carefully regulated to ensure that they are initiated only when appropriate, and efficiently resolved upon pathogen eradication, or when the burden of tissue destruction outweighs the benefit of pathogen control. Indeed, defects in T_{Reg} cell function can result in T_H1-, T_H2- or T_H17-mediated inflammatory disease, indicating that these cells are required for the proper regulation of multiple types of immune responses.

Several recent studies have demonstrated that T_{Reg} cells use canonical T_H cell-associated transcription factors in order to maintain or restore immune homeostasis during polarized T_H1-, T_H2- and T_H17-driven immune responses. For instance, in addition to controlling the differentiation, migration and function of IFNγ-producing T_H1 cells, T-bet influences the generation of effector and memory $CD8^+$ T cells, and regulates the homeostasis and function

of natural killer (NK) cells, thereby coordinating the cellular immune response to intracellular infection.[40, 44–46]. Interestingly, T-bet is also expressed by a subset of T_{Reg} cells, and is required for T_{Reg} cell homeostasis and function during polarized T_H1 inflammatory responses[47]. T-bet$^+$ T_{Reg} cells accumulate at sites of T_H1-type inflammation, and T-bet deficient- T_{Reg} cells display impaired proliferation during T_H1-mediated immune responses, ultimately failing to control expansion of IFNγ-producing T_H1 cells when transferred into FOXP3-deficient *scurfy* mice.

Similarly, T_{Reg} cell expression of interferon regulatory factor 4 (IRF4), a transcription factor involved in control of IL-4 production by CD4+ T cells and T_H2 differentiation, is required for T_{Reg} cell mediated-control of T_H2-type inflammatory responses[48]. Mice in which IRF4 is specifically deleted within FOXP3$^+$ T_{Reg} cells develop a lymphoproliferative disease associated with a selective increase in the number and frequency of IL-4- and IL-5-producing CD4$^+$ T cells and have elevated serum levels of IgG1 and IgE. Consistent with this increase in serum antibodies, these animals also contain dramatically increased numbers of plasma cells and spontaneously develop splenic germinal centers. Interestingly, IRF4 is also critical for the differentiation of T follicular helper cells (T_{FH}), which provide help to B cells and help regulate antibody production *in vivo*[49]. Coupled with the dearth of eosinophilia and IL-13-producing CD4$^+$ T cells, the profound increase in germinal center formation in these animals implies that T_{Reg} cell expression of IRF4 may be required to control a specific component of T_H2-associated inflammation, namely aberrant T_{FH} activation and high affinity antibody production. Finally, deletion of the transcription factor signal transducer and activator of transcription (STAT3) in T_{Reg} cells results in development of spontaneous fatal intestinal inflammation triggered by excessive IL-17 production without significant differences in T_H1- or T_H2-associated inflammatory cytokines, indicating a selective dysregulation of T_H17 responses by STAT3-deficient T_{Reg} cells[50].

The mechanisms by which T-bet, IRF4 and STAT3 control T_{Reg} cell activity during T_H1, T_H2 and T_H17 responses are still unclear, but likely involve a combination of influences on T_{Reg} cell migration, function and homeostasis. For instance, T_{Reg} cells deficient in T-bet, IRF4 or STAT3 display impaired expression of chemokine receptors implicated in T_{Reg} cell localization during T_H1- (CXCR3), T_H2- (CCR8) or T_H17- (CCR6) mediated inflammation, suggesting that altered T_{Reg} cell migration may therefore underlie some of the functional defects in these cells[47, 48, 50]. Additionally, loss of these transcription factors may impact the functional properties of T_{Reg} cells. Of note, T_{Reg} cells lacking T-bet, IRF4 or STAT3 all show reduced expression of *Il10*[47, 48, 50]. Moreover, IRF4 and STAT3-deficient T_{Reg} cells have reduced expression of other genes associated with T_{Reg} cell function such as *Icos*, *Fgl2*, *Ebi3* (which encodes a component of the immunosuppressive cytokine IL-35), *Prf1* and G*zb*[48, 50]. By contrast, expression of *Ctla4* is substantially increased in STAT3-deficient T_{Reg} cells, while *Tgfb1* expression was unaltered in T_{Reg} cells lacking any of the aforementioned transcription factors. Finally, loss of T-bet expression resulted in the impaired proliferation and accumulation of T_{Reg} cells during T_H1-type inflammatory responses and the failure of T-bet-deficient T_{Reg} cells to control T_H1-driven inflammatory responses may in part be secondary to the inability of these cells to survive and proliferate in a highly polarized T_H1-type environment[47]. From these data, a model emerges whereby selective expression or activation of transcriptional regulators associated with specific T_H1, T_H2 and T_H17 cells drives the phenotypic and functional specialization of T_{Reg} cells, endowing them with the molecular machinery needed to restrain these different types of CD4$^+$ T cell responses (Figure 2). This model has important implications for use of T_{Reg} cells therapeutically, as it implies that only specific subsets of T_{Reg} cells will be efficacious in treating T_H-1, T_H2- or T_H17-driven inflammatory diseases. Thus, it will be critical in future studies to identify and characterize the cellular and molecular mechanisms that

underlie the functional specialization of T_{Reg} cells, and to develop methods for selectively isolating and expanding different subsets of T_{Reg} cells.

Environmental control of T_{Reg} cells

The need to carefully modulate both the number and activity of T_{Reg} cells to maintain the balance between autoimmunity and immunosuppression suggests that T_{Reg} cells pay close attention to the immune environment, and alter their phenotype, migration and function in response to specific cues encountered in the periphery. Cytokines are a diverse group of generally secreted proteins that control nearly all aspects of leukocyte biology. They do so by binding to specific receptors on the surface of cells which transmit signals promoting cellular proliferation, activation, differentiation and/or death. Broadly speaking, cytokines can be divided into those that are constitutively expressed and promote normal lymphocyte development and homeostasis, and those whose expression is induced by specific inflammatory stimuli. Decades of research have demonstrated that both homeostatic and inflammatory cytokines have a profound influence on the phenotypic and functional differentiation of the various T cell populations. In addition to cytokines, non-protein small molecules such as steroids, sphingolipids and metabolites of vitamins A and D can also influence T cell differentiation, migration and function[51–54]. In the following sections, we will discuss recent progress in understanding how T_{Reg} cells respond to the cytokines, vitamin metabolites and other factors present in the immune environment, and how this controls their activity in health and disease (Figure 3).

Control of T_{Reg} cell homeostasis

Common-γ chain-signalling cytokines

By far, the best studied cytokine in terms of its impact on the development, homeostasis and function of T_{Reg} cells is IL-2[55]. Indeed, T_{Reg} cells were initially identified based on their constitutive expression of the high-affinity IL-2 receptor component CD25. Moreover, it is clear that IL-2 has an essential and non-redundant role in controlling T_{Reg} cell function in the periphery, as evidenced by the lymphoproliferative disease and colitis that develop in either IL-2- or CD25-deficient mice. However, the precise manner by which IL-2 influences T_{Reg} cell function is still largely unknown. Several studies have demonstrated that T_{Reg} cells occupy a distinct homeostatic ‘niche’, and this is thought in part to be controlled by access to IL-2[56, 57]. Accordingly, blocking IL-2 activity through the administration of IL-2-specific antibodies to mice decreased proliferation of $CD4^+CD25^+$ T cells, and impaired T_{Reg} cell activity[58]. These data led to a model in which IL-2 produced by activated $CD4^+FOXP3^-$ T cells acts in a paracrine fashion to promote T_{Reg} cell proliferation and survival. However, both the frequency and absolute number of peripheral T_{Reg} cells are largely normal in mice lacking either IL-2 or CD25, indicating that the homeostasis of T_{Reg} cells is more complicated than previously appreciated[59, 60] (Box 2). In addition, expression of CD25 varies substantially among different T_{Reg} cell populations, with activated $CD44^{hi}$ T_{Reg} cells generally being $CD25^{low/-}$[21]. Interestingly, nearly all T_{Reg} cells in IL-2- and CD25-deficient mice belong to the $CD44^{hi}$ subset, and have increased expression of other activation markers such as CD69, CD103 and inducible T cell co-stimulator (ICOS)[57]. Although the activated phenotype of T_{Reg} cells in these mice is thought to be secondary to their inflammatory disease, an alternate interpretation of these results is that the critical function of IL-2 is to help sustain the $CD44^{lo}CD25^{hi}$ T_{Reg} cell subset, and that this population has a non-redundant function in preventing lymphoproliferative and autoimmune disease.

The abundance of functional $CD44^{hi}$ T_{Reg} cells in IL-2- and CD25-deficient indicates that either the maintenance of these cells does not normally depend on IL-2, or that other cytokines can compensate for loss of IL-2 in driving the homeostasis of this population. Like

IL-2, IL-15 uses the common-γ chain and CD122 as the signalling components of its receptor. As such, they are thought to deliver very similar signals into cells, and thus can have redundant functions. For instance, thymic development of T_{Reg} cells proceeds normally in mice lacking IL-2, IL-15, CD25 or IL15Rα, but is severely disrupted in either CD122-deficient or IL-2/IL-15 double-deficient mice[59, 60]. In the periphery, IL-15 is 'trans-presented' in association with IL15Rα on the surface of DCs, where it supports the homeostasis of $CD8^+$ memory T cells and NK cells[61]. As previously discussed, T_{Reg} cells functionally interact with DCs in lymphoid tissues[29, 30], and this may grant them access to trans-presented IL-15 that could help support their peripheral survival and function. However, the precise role of IL-15 in the IL-2-independent peripheral homeostasis of different T_{Reg} cell populations has not been explored.

Vitamin A and D metabolites

At barrier tissues such as the intestine and skin, the immune system faces the daunting task of ignoring the large number of commensal organisms and harmless environmental antigens while responding vigorously to enteric and cutaneous pathogens. Recent studies have demonstrated that metabolites of vitamins A and D can help control this balance in by influencing the development and homeostasis of cutaneous and intestinal T_{Reg} cells. For example, retinoic acid produced from dietary vitamin A induces T_{Reg} cell expression of the intestinal homing receptors α4β7 integrin and CCR9[62]. Additionally, retinoic acid promotes the peripheral differentiation of inducible T_{Reg} cells, and helps sustain T_{Reg} cell numbers and function during inflammatory responses[63–66]. Among DCs, the enzymes that convert vitamin A into retinoic acid are most prominently expressed in a population of $CD103^+$ DCs found in the intestine and the gut-associated lymphoid tissues[64, 65]. Thus, under steady state conditions, retinoic acid produced by these DCs is thought to influence the balance of effector and regulatory T cells in the intestine, and help promote tolerance to commensal bacteria and food antigens.

Although retinoic acid is primarily thought to promote T_{Reg} cell activity in the intestine, the active form of vitamin D, 1,25-Dihydroxyvitamin D3, can have similar effects in the skin, where vitamin D is produced in response to sunlight. Like retinoic acid, 1,25-Dihydroxyvitamin D3 can both augment the function of existing T_{Reg} cells and promote *de novo* differentiation of T_{Reg} cells from naive $CD4^+$ T cell precursors[54, 67]. Moreover, 1,25-Dihydroxyvitamin D3 produced by cutaneous DCs can induce T cell expression of CCR10, a chemokine receptor implicated in T cell localization to the epidermis[68], although this has not been formally demonstrated in T_{Reg} cells. Nonetheless, these data raise the intriguing possibility that anatomical cues delivered by metabolites enriched in the skin vs. intestine can drive T_{Reg} cell specialization, endowing them with the migratory and functional properties needed for immunoregulation in these tissues.

Inflammatory cytokines and T_{Reg} cells

During infection, cytokines produced in response to pathogen recognition by cells of the innate immune system initiate the inflammatory response, which is subsequently amplified by products of the adaptive immune response. These inflammatory cytokines have broad effects on the phenotypes, functions and migration of T cells and other leukocyte populations, and ultimately dictate the course of the pathogen eradication. Additionally, dysregulated production of inflammatory cytokines underlies the pathogenesis of most autoimmune and inflammatory diseases. Because of their central function in driving inflammatory responses, it is not surprising that many of these cytokines can also act directly on T_{Reg} cells, influencing their phenotype and activity in complex ways such that vigorous immune responses are allowed to occur when necessary, but generally with the restraint needed to prevent collateral damage and immunopathology. Indeed, this complexity

is only beginning to be appreciated and addressed experimentally, and although not exhaustive, the following discussion highlights the fact that many cytokines can have both positive and negative effects of T_{Reg} cell activity. Thus, careful analyses are still required to determine how T_{Reg} cell activity is augmented and inhibited by various inflammatory cytokines during different types of inflammatory responses, and this promises to be a fruitful area of future study that has substantial implications for the development of therapies aimed at manipulating T_{Reg} cell activity.

IFN-γ and IL-12

IFNγ and IL-12 function together to promote T_H1 cell differentiation and function[69]. Additionally, IFNγ is the principle effector cytokine produced by T_H1 cells, NK cells and $CD8^+$ T cells, and it is required for clearance of intracellular pathogens such as *Mycobacterium tuberculosis, Leishmania major* and *Listeria monocytogenes*[70–72]. Like many inflammatory cytokines, IFNγ actively inhibits the peripheral generation of $FOXP3^+$ T_{Reg} cells from naive $CD4^+$ cells[73]. However, IFNγ signalling via STAT1 activation also drives T-bet expression by thymus-derived T_{Reg} cells, and as discussed in the preceding section, this endows them with the molecular machinery required for efficient control of T_H1-type responses[47]. Thus, in addition to being a pro-inflammatory cytokine essential for combating intracellular pathogens, IFNγ also has an immunoregulatory function that may help limit the magnitude and duration of T_H1-type inflammatory responses. However, although IFNγ-induced signalling within T_{Reg} cells may be beneficial for the differentiation of functionally specialized T-bet^+ T_{Reg} cells, excessive STAT1 activation can have a deleterious effect on T_{Reg} cell function, resulting in excessive T_H1 cell activity and inflammatory disease[74]. Similarly, a recent study demonstrated that when IL-2 availability is limited during oral infection with *Toxoplasma gondii*, T_{Reg} cells become IL-12 responsive, express high amounts of T-bet and acquire the ability to produce IFNγ, subsequently contributing to the fatal intestinal immunopathology that develops during infection[75]. Thus, IFNγ and IL-12 can either promote or inhibit T_{Reg} cell activity depending on the magnitude of the cytokine response and the context in which it is perceived. These studies emphasize the need to precisely regulate T_{Reg} cell responses to these cytokines during T_H1-mediated inflammation, and underscore the complex and confounding impact cytokines can have on T_{Reg} cell activity *in vivo*.

IL-6

IL-6 is a widely expressed cytokine with multiple functions that can have a profound influence on T_{Reg} cell development and activity[76, 77]. For instance, IL-6 potently prevents the TGFβ-mediated development of inducible T_{Reg} cells, and instead acts with TGFβ to induce T_H17 cell differentiation[78]. Thus, the presence or absence of IL-6 can regulate the induction of proinflammatory and tolerogenic T cell responses, respectively. Moreover, in addition to controlling the development of inducible T_{Reg} cells, IL-6 can also influence the stability and function of thymus-derived T_{Reg} cells. For instance, stimulation of T_{Reg} cells in the presence of IL-6 results in loss of FOXP3 expression and acquisition of a T_h17 cell phenotype and function[79, 80]. Moreover, IL-6 produced downstream of Toll-like receptor ligation blocks T_{Reg} cell-mediated inhibition of T cell activation, and this is likely due to both direct effects of IL-6 on T_{Reg} cells, as well as the ability of IL-6 to render effector T cells resistant to T_{Reg} cell-mediated suppression[81].

Although these studies emphasize the ability of IL-6 to inhibit T_{Reg} cell activity, the importance of STAT3 for T_{Reg} cell-mediated control of T_h17 responses raises the intriguing question; what are the important STAT3-activating cytokines that act on T_{Reg} cells? IL-6 is a potent activator of STAT3, and thus it is tempting to speculate that as with IFNγ and STAT1 during T_H1 cell responses, IL-6 simultaneously promotes T_H17 cell differentiation

while acting on T_{Reg} cells via STAT3 to maximize their ability to modulate T_H17 responses. However, several other cytokines also activate STAT3, including IL-10, IL-27 and IL-21, and the impact of these cytokines on T_{Reg} cell activity during T_h17 responses remains to be determined. Nonetheless, it is clear that the presence or absence of IL-6 can modulate T_{Reg} cell activity during inflammatory responses. As drugs targeting IL-6 reach the clinic[82], it will be interesting to determine how IL-6 blockade impacts the abundance, phenotype and functional activity of T_{Reg} cells in the context of immune-mediated diseases such as rheumatoid arthritis and inflammatory bowel disease.

IL-4

IL-4 is a key effector cytokines produced by T_h2 cells, and is also required for T_h2 cell differentiation. However, although the effects of IL-4 on TH2 cell differentiation and function are well characterized, its impact on T_{Reg} cell stability and function are not well understood. Indeed, like many other inflammatory cytokines, IL-4 can both augment and inhibit T_{Reg} cell development and function in different experimental settings. For instance, similar to IL-6, IL-4 can inhibit TGFβ-induced peripheral T_{Reg} cell development, and act with TGFβ to drive development of a recently described population of IL-9-producing effector T cells[83, 84]. Additionally, IL-4 is thought to render T_h2 cells resistant to T_{Reg} cell-mediated suppression[85]. By contrast, IL-4 stimulation of T_{Reg} cells can boost their expression of CD25 and FOXP3, prevent their apoptosis and increase their suppressive function *in vitro*[85, 86]. Thus, the effects of IL-4 on T_{Reg} cells clearly require further investigation, and likely depend on other factors such as the presence or absence of TGFβ in the inflammatory environment.

Type-1 IFNs

The type-1 IFNs are a group of more than a dozen closely related cytokines that are highly upregulated during viral infection and function to block viral replication and to qualitatively and quantitatively influence the anti-viral adaptive immune response. However, overproduction of type-1 IFN has been associated with a variety of autoimmune disorders[87]. Surprisingly, despite the dramatic effects type-1 IFNs can have on conventional $CD4^+$ and $CD8^+$ effector T cells, and the clear association between type-1 IFN production and development of autoimmunity, the effects of type-1 IFNs on the development, homeostasis and function of T_{Reg} cells are largely unexplored. Several studies have indicated that IFNβ treatment can help restore both the number and function of T_{Reg} cells in patients with multiple sclerosis[88, 89]. By contrast, stimulation with IFNα inhibited T_{Reg} cell generation in an *in vitro* culture system, though this was due largely to inhibition of IL-2 production by effector T cells[90]. Clearly, more careful studies are needed to determine how type-1 IFNs impact T_{Reg} cell homeostasis and function, and how this in turn modulates T_{Reg} cell activity during acute and chronic viral infection, and in type-1 IFN-associated autoimmune diseases.

TNF and IL-1

Tumour necrosis factor (TNF) and IL-1 are both pleiotropic cytokines that act on a wide range of cells and generally promote inflammation. Indeed, both TNFα and IL-1 have been successfully targeted therapeutically for the treatment of a number of inflammatory and autoimmune diseases, including rheumatoid arthritis, psoriasis and inflammatory bowel disease[91]. It is not surprising therefore that these cytokines can significantly impact T_{Reg} cell function. Although TNFα is primarily known for its proinflammatory functions, most current evidence indicates that upon binding and signaling through TNFR2, this cytokine actually potentiates T_{Reg} cell activity[92]. Indeed, TNFR2 is expressed by a subset of effector or memory phenotype T_{Reg} cells that are highly suppressive, and *in vitro* treatment of mouse T_{Reg} cells with TNFα can augment their proliferation and suppressive activity[93]. Furthermore, blockade of TNFα actually exacerbates cutaneous inflammation in a mouse

model of psoriasis, and this was associated with decreased expansion of populations of T_{Reg} cells[94]. Simialry, TNFα–TNFR2 interactions may control T_{Reg} cell population expansion during cecal ligation and puncture (CLP) in mice[93], although the requirement for direct recognition of TNFα by T_{Reg} cells was not addressed in either the psoriasis or CLP studies. The impact of IL-1 on T_{Reg} cell function is still poorly understood. Although IL-1 could potentiate expansion of $FOXP3^+$ cells from cultures of $CD4^+CD25^+$ cells, this was due to the effects of IL-1 stimulation of $FOXP3^-$ cells, and IL-1 did not directly augment proliferation of $FOXP3^+$ cells[95]. In fact, in conjunction with IL-2, IL-1 acts to convert T_{Reg} cells into $FOXP3^-$ T_H17 cells[96].

Stability of Treg cells

The ability of cytokines such as IL-1, IL-6 and IL-12 to downregulate FOXP3 expression and convert T_{Reg} cells into proinflammatory effector cells suggests that T_{Reg} cells retain some functional plasticity. Indeed, a subject of recent controversy is the extent to which T_{Reg} cells can extinguish forkhead box P3 (FOXP3) expression and convert to conventional $FOXP3^-$ effector T cells. T_{Reg} cells induced by TGF-β in the periphery show incomplete demethylation of the *Foxp3* locus that is associated with unstable Foxp3 expression[97]. However, several studies have demonstrated that even thymic-derived T_{Reg} cells can convert to an effector phenotype following transfer into lymphopenic recipients[98–100], and during *ex vivo* stimulation with inflammatory cytokines[79, 80, 96].

To determine if a portion of T_{Reg} cells converts to an effector phenotype in lymphoreplete mice, two groups recently generated mice engineered to track the fate of $FOXP3^+$ cells *in vivo*. In these systems, mice expressing the cre recombinase in T_{Reg} cells under control of regulatory elements from the *Foxp3* gene were crossed to mice in which cre-mediated recombination removes a stop codon in a fluorescent reporter protein knocked in to the ubiquitously expressed *Rosa26* locus. Thus, in these animals, cells even transiently expressing Foxp3 are permanently marked, and their phenotypic and functional properties can be spatially and temporally examined. Using this system, Bluestone and colleagues observed that a portion of $FOXP3^+CD4^+$ cells downregulate FOXP3 expression and acquire the ability to produce effector cytokines such as interferon-γ (IFNγ) even in the absence of any experimental manipulation. Furthermore, the frequency of these 'ex- T_{Reg} cells' was increased in the pancreatic islets of non-obese diabetic (NOD) mice, indicating that they may contribute to autoimmune pathology[101]. These data are complemented by another recent study demonstrating that during infection with a lethal strain of *Toxoplasma gondii*, T_{Reg} cells can lose FOXP3 expression and acquire T_H1 effector characteristics[75]. Together, these studies suggest that highly polarized inflammatory environments can subvert T_{Reg} cell function by converting them to $FOXP3^-$ effector T cells *in vivo*, and are consistent with a recent epigenetic analysis demonstrating that the loci encoding key transcription factors and cytokines associated with T_H1, T_H2 and T_H17 cells are not fully repressed in T_{Reg} cells[102]. However, using a similar reporter mouse system to monitor T_{Reg} cell stability, Rudensky's group recently demonstrated that FOXP3 expression by T_{Reg} cells is remarkably stable, even in highly inflammatory settings[103]. The discrepancies in these studies may be due to differences in the inflammatory systems used to examine T_{Reg} cell stability *in vivo*, or to subtle differences in the way the reporter mice were constructed. Bluestone's group drove cre expression using a bacterial artificial chromosome (BAC) transgene, whereas Rudensky's group knocked a cre expression cassette into the 3' untranslated region of the endogenous Foxp3 locus, and this may lead to differences in the timing and extent of cre expression that result in the divergent conclusions of these studies.

In discussing the stability of T_{Reg} cells, it is also important to keep in mind differences in Foxp3 expression observed in mouse vs. human T cells. In mouse, Foxp3 appears to be a

robust marker for either thymic-derived or induced T_{Reg} cells. However, nearly all human $CD4^+$ T cells transiently express Foxp3 during activation, and this is not associated with acquisition of regulatory function. Thus, Foxp3 alone is not a reliable marker for human T_{Reg} cells, further complicating analyses of their function and stability. Moreover, because thymic output is severely curtailed following puberty, causing fewer T_{Reg} cells to emerge from the thymus, less-stable induced T_{Reg} cells may assume a greater role in maintaining immune homeostasis in adults[104]. Clearly, the degree to which T_{Reg} cells can acquire effector functions and contribute to the development of autoimmune and inflammatory diseases merits further study, with careful attention payed to potential differences observed in mouse and systems.

Concluding Remarks

T_{Reg} cells have emerged as potent anti-inflammatory cells, and this has generated considerable excitement for the potential to therapeutically manipulate T_{Reg} cell activity to treat autoimmune disease, prevent graft rejection, and boost immune responses during cancer and chronic infection[105]. However, it is now evident that T_{Reg} cells are a dynamic and diverse T cell population, composed of several phenotypically and functionally distinct subsets whose differentiation and function are controlled by specific signals in the immune environment. Thus, in order to optimally utilize T_{Reg} cells in the clinic, it is essential that we better understand how these subsets function together to ensure that robust immune responses can occur when needed without development of significant immunopathology and inflammatory disease. Key unresolved issues include defining the importance of each T_{Reg} cell subset in the control of different types of inflammatory responses, identifying the factors that govern the peripheral differentiation of these subsets, and determining how cytokines and other factors in the immune environment influence T_{Reg} cell activity and stability during the initiation, progression and termination of normal and pathological immune responses.

BOX 1: Influence of the T cell receptor on T_{Reg} cell phenotype and function

Thymically-derived regulatory T (T_{Reg}) cells are thought to be largely autoreactive[106]. However, the identity of the natural self-antigens thought to drive T_{Reg} cell development is still completely unknown. Despite the fact that T_{Reg} cell specificity is still poorly characterized, there is evidence that T cell receptor (TCR) recognition has a key role in influencing the phenotype, function and localization of T_{Reg} cells *in vivo*. For instance, activation of antigen-specific T_{Reg} cells isolated from TCR transgenic mice alters their expression of several lymphocyte homing receptors, resulting in their subsequent redistribution to non-lymphoid tissues[10, 19]. Moreover, T_{Reg} cells isolated from different tissues have distinct TCR repertoires, indicating that TCR-driven activation helps impart cells with tropism for specific sets of lymphoid and non-lymphoid tissues[107]. This likely occurs as T_{Reg} cells specific for tissue-restricted self-antigens encounter signals during their activation that drive expression of tissue-specific homing receptors[62]. Although together these data suggest a key role for TCR recognition of specific self-antigens in driving the phenotypic diversity present among T_{Reg} cells, a better understanding of the self-antigens recognized by T_{Reg} cells is necessary to clarify precisely how their specificity impacts their localization, phenotype, homeostasis and function.

BOX 2: T_{Reg} cell homeostasis – beyond IL-2

Lymphocyte homeostasis is the process by which various T and B cell populations are maintained at near constant frequencies in the periphery due to their balanced generation, proliferation and death. The major cytokine thought to control T_{Reg} cell homeostasis is interleukin-2 (IL-2). However, many other factors have been identified that influence

T_{Reg} cell homeostasis. For instance, T_{Reg} cell expression of the co-stimulatory molecule CD28 is required for their peripheral maintenance, and T_{Reg} cell numbers are substantially reduced in CD28-deficient mice[108]. Accordingly, these animals show enhanced susceptibility to autoimmune diabetes[109]. Given that T_{Reg} cells form long-lasting contacts with DCs in the secondary lymphoid tissues, it is also not surprising that T_{Reg} cell proliferation and abundance are highly sensitive to changes in DC frequency[110]. This is likely due to the ability of DCs to present self-antigens to T_{Reg} cells, and to provide CD28-dependent co-stimulatory signals via CD80 and CD86 that can drive T_{Reg} cell activation and proliferation. Another molecule that influences T_{Reg} cell homeostasis is sphingosine 1-phosphate (S1P). S1P is best known for promoting lymphocyte egress form the thymus, spleen and LNs. However, T_{Reg} cells overexpressing the S1P receptor, $S1P_1$, have a competitive disadvantage in the periphery, suggesting that S1P can also modulate the proliferation and/or survival of T_{Reg} cells via activation of the AKT–mTOR (mammalian target of rapamycin) pathway[51]. Other molecules implicated in T_{Reg} cell homeostasis include CD44, which can promote T_{Reg} cell proliferation upon binding to high molecular weight hyaluronan in the extracellular matrix[111], and CC-chemokine receptor 4 (CCR4), which is required for the proper homeostasis of $CD103^{hi}$ cutaneous T_{Reg} cells[10]. Thus, T_{Reg} cell homeostasis is far more complex than currently appreciated, and much more work needs to be done to understand how T_{Reg} cell abundance is controlled in various tissues, both in the steady-state and during the course of different types of immune responses.

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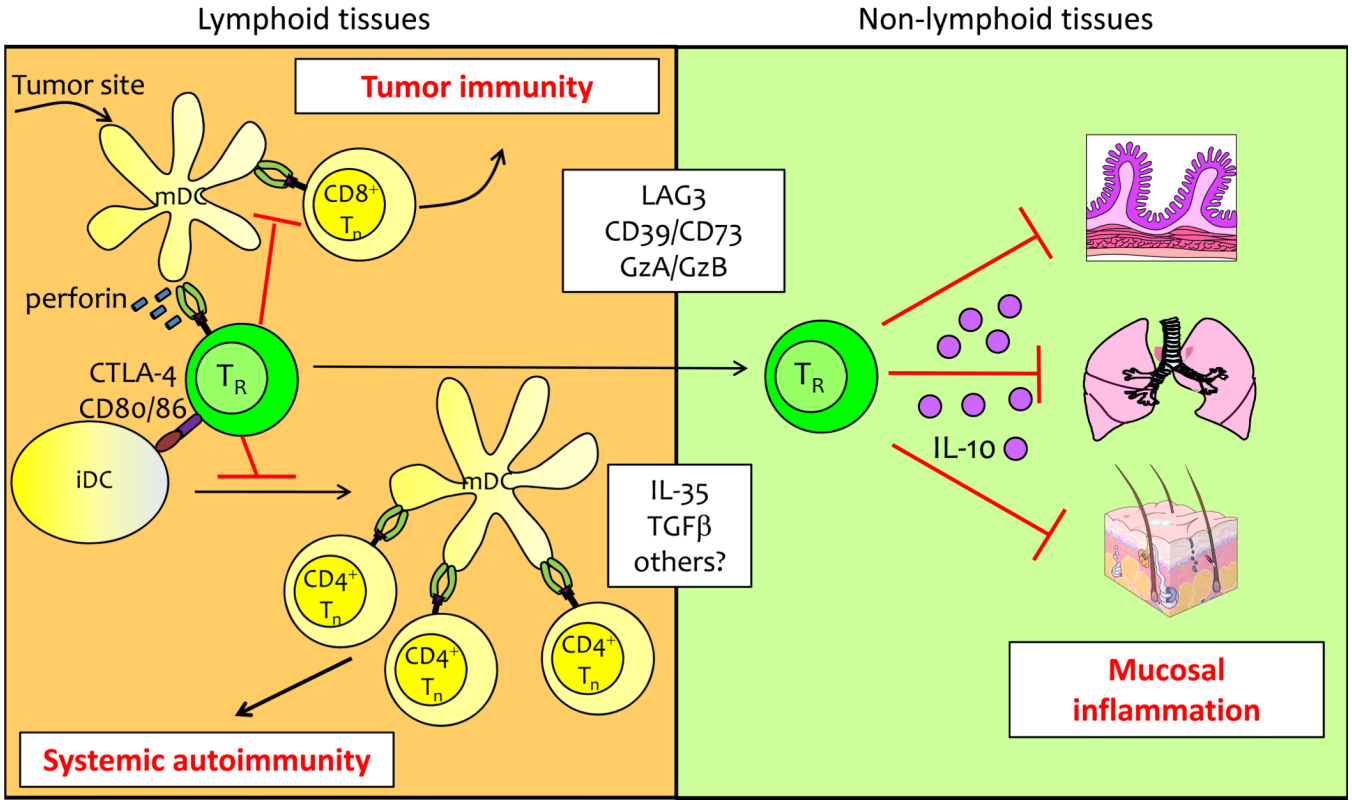

Figure 1. Differing immunosuppressive mechanisms used by T_{Reg} cells in lymphoid vs. non-lymphoid tissues

(Left) T_{Reg} cells in secondary lymphoid tissues use multiple mechanisms to inhibit DC function and block initiation of autoimmunity or prevent tumor clearance. (Right) T_{Reg} cell production of IL-10 is essential for immunoregulation at mucosal tissues such as the intestines, lungs and skin. The relative importance of other immunosuppressive mechanisms used by T_{Reg} cells (shown in white box) in lymphoid vs. non-lymphoid tissues remains to be established

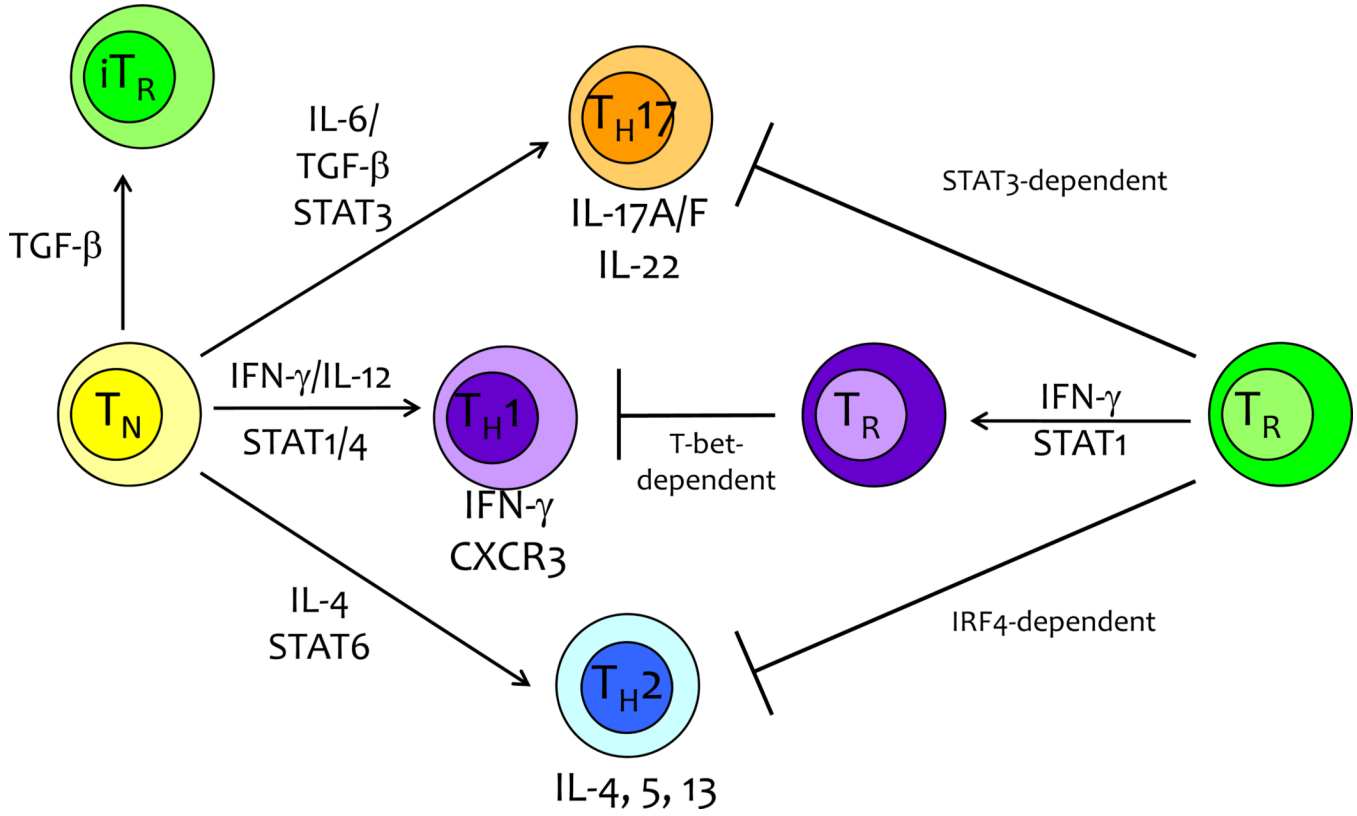

Figure 2. Functional Differentiation of T_{reg} cells and T_{conv} cells

The differentiation of naive T_H cells into functionally distinct effector subsets (T_H17, T_H1, T_H2, induced T_R) is dependent on the induction of key transcriptional regulators (RORγT, T-bet, GATA3, Foxp3) following TCR stimulation in conjunction with cytokine signaling/ STAT activation. Comparably, thymic-derived T_{reg} cells utilize specific molecular programs driven by STAT3, T-bet, or IRF4 to restrain particular types of immune responses orchestrated by distinct effector T cell subsets.

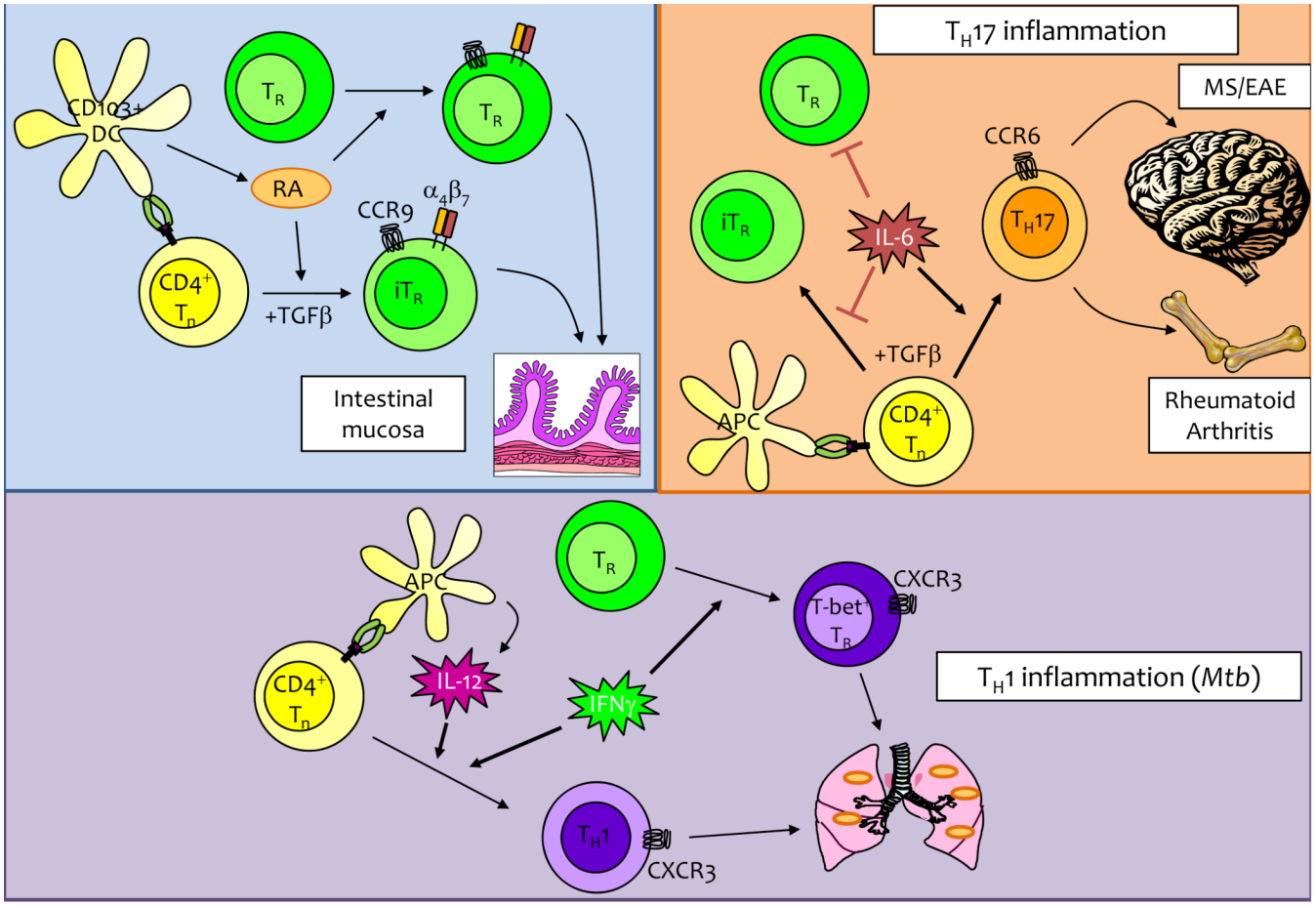

Figure 3. Modulation of T_{Reg} cell activity can be by different factors in the immune environment

(Left) The vitamin-A metabolite retinoic acid (RA) can boost T_{Reg} cell activity within the intestine by inducing Foxp3 expression in naïve T cells and directing the expression of gut-homing receptors on both T_{Reg} and iT_{Reg} cells. (Right) During T_H17-driven inflammation, IL-6 impairs T_{Reg} cell activity both by directly blocking T_{Reg} function as well as by promoting T_H17 differentiation at the expense of iT_{Reg} generation. (Middle) During type-1 inflammatory responses, such as *Mtb* infection, IFNγ and IL-12 direct T_H1 differentiation. Coordinately, IFNγ produced in response to infection directs the differentiation of T-bet$^+$ T_{Reg} cells that are specialized to restrain pro-inflammatory T_H1 cells.

Table 1

A summary of important homing receptors expressed by T_{Reg} cells and their functions.

Site of T_{Reg} cell migration	Receptor	Function	Key References
Lymphoid tissues	CD62L	Migration to lymph nodes	Function in T_{Reg} cell migration to lymph nodes[112] Importance in NOD/SCID model of diabetes[113]
	CCR7	Migration to lymph nodes and spleen	$CCR7^{-/-}$ T_{Reg} cells fail to prevent colitis[14] Importance in T_{Reg} cell migration during allograft rejection[18]
Non-lymphoid tissues	E-/P-selectin ligand	Migration to skin and other inflamed tissues	Expression by human T_{Reg} cells[114] Function in cutaneous hypersensitivity response[115] Importance in cutaneous tolerance[11]
	αEβ7 integrin	Epithelial localization	Expression by mouse T_{Reg} cells[20] T_{Reg} cell retention during infection with *Leishmania major* [12]
	α4β7 integrin	Migration to gut-associated lymphoid tissues	Function in T_{Reg} cell migration to the intestine[116]
	CCR2	Migration to inflamed tissues	Expression by human and mouse T_{Reg} cells[19, 117] Importance in T_{Reg} cell migration during allograft rejection[18]
	CCR4	Migration to skin and other inflamed tissues	Expression by human T_{Reg} cells[114] Importance in cutaneous/pulmonary tolerance[10] $CCR4^{-/-}$ T_{Reg} cells fail to prevent colitis[118]
	CCR5	Migration to inflamed tissues	Importance in T_{Reg} cell migration during allograft rejection[18] Function in migration to the inflamed intestine[119] Directing T_{Reg} cell localization infection with *Leishmania major*[120]
	CCR6	Migration to sites of T_H17-mediated inflammation	Expression by IL-10-producing T_{Reg} cells[121] Function in T_{Reg} cell migration during Th17-mediated autoimmunity[15, 122]
	CCR8	Migration to skin and sites of Th2-mediated inflammation	Expression by T_{Reg} cells[123]
	CCR9	Migration to small intestine	Expression by T_{Reg} cells in the intestinal lamina propria[124]
	CCR10	Migration to mucosal tissues and skin	Expression by T_{Reg} cells in inflamed human liver[125]
	CXCR3	Migration to sites of T_H1-mediated inflammation	Expression by T_{Reg} cells is T-bet dependent[47] Importance in T_{Reg} cell localization to inflamed liver[16, 126]
	CXCR5	Migration to B cell follicles and germinal centers	Expression by human T_{Reg} cells[127] Inhibition of B cell responses by T_{Reg} cells[128]
	CXCR6	Migration to the liver	Expression by human T_{Reg} cells[127]
Both lymphoid and non-lymphoid tissues	CXCR4	Migration to bone marrow, Peyer's patches and tumour sites	Expression by naïve phenotype T_{Reg} cells[127] Association with tumor infiltrating T_{Reg} cells[129, 130]