Homeostatic control of regulatory T cell diversity

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Abstract

Regulatory T (Treg) cells constitute an essential counterbalance to adaptive immune responses. Failure to maintain appropriate Treg cell numbers or function leads to autoimmune, malignant and immunodeficient conditions. Dynamic homeostatic processes preserve FOXP3[+] Treg cells within a healthy range, with high rates of cell division offset by apoptosis under steady-state conditions. Recent studies have shown how Treg cells specialise in different environmental contexts, tailoring their functions and homeostatic properties to adapt to a wide variety of tissues and immune conditions. In this review, we provide new insights into the molecular controls that maintain the steady-state homeostasis of Treg cells and the cues that drive Treg cell adaptation to inflammation and/or different locations. We highlight how differing local milieu may drive context-specific Treg cell function and restoration of immune homeostasis, and how dysregulation of these processes can precipitate disease.

Introduction

FOXP3$^+$ regulatory (Treg) cells are a suppressive subset of CD4$^+$ T cells that act to antagonise immunity. With the capacity to prevent both potentially damaging autoimmune and protective immune responses, the number of Treg cells is a critical determinant of the regulatory burden on the immune system. Too few Treg cells can trigger fatal autoimmune responses, while too many can cause immune suppression.

The suppressive function of Treg cells is directed by the transcription factor FOXP3. The expression of high levels of FOXP3 and an epigenetically modified *Foxp3* locus is associated with suppressive function in both human and mouse CD4$^+$ T cells, although transient low levels of FOXP3 expression can occur without imparting the Treg cell phenotype[1]. Induction of FOXP3 expression occurs in a non-random manner, with Treg cells having a distinct T cell receptor (TCR) repertoire that is thought to have increased affinity with self-antigen compared with conventional CD4$^+$ T cells[2]. The thymus is a major site of Treg cell induction, where FOXP3 expression is initiated through a combination of antigen recognition and microenvironmental influences[2]. However, it should be noted that FOXP3$^-$ recent thymic emigrants can be preferentially induced to express FOXP3[3,4]; thus "thymic Treg cells" are probably a mixture of true thymus-generated Treg cells and Treg cells induced in the periphery from recent thymic emigrants. In addition, peripheral induction of FOXP3 can occur in various tissues, including the colon[5] and the placenta[6], sites where Treg cells of this origin are important to preserve immune homeostasis[7]. The relative proportion of thymic Treg cells remains unknown, but is estimated at 70-90% using the markers Helios and Neuropilin 1, which are enriched within this population[8-10].

Contrary to the original conception of Treg cells as a generic level of immune regulation, Treg cells are neither monolithic nor static in nature. In addition to distinctions based on the location of induction ("thymic" or "peripheral"), Treg cells can be divided into functional subsets. These include a "central" population, which have circulatory characteristics that are similar to naïve conventional CD4$^+$ T cells, several "effector" populations, which have enhanced function and signs of recent antigen encounter, and polarised tissue-resident populations, which are present in most non-lymphoid organs. Furthermore, the Treg cell population has a very high turnover and exquisite sensitivity to a range of signals from their environment. These properties impart the capacity for the rapid adjustments in their number, location and function that are required to effectively react to immune dynamics. In this Review, we explore the recent advances in our knowledge about the diversity of Treg cell populations, how this diversity is maintained throughout homeostasis and immune challenge, and the diseases that occur when Treg cell homeostasis is impaired.

Heterogeneity of the circulating Treg cell population

"Central" and "Effector" Treg cells

The specification and function of Treg cells is imparted by the transcription factor, FOXP3. Continued expression of FOXP3 is required to maintain this program and suppressive function in the periphery[11]. However, FOXP3 does not act alone. Several other transcription factors can and do cooperate with FOXP3 to stabilise expression of the Treg cell signature[12-14]. In addition to directing expression of Treg cell signature genes (such as *Il2ra*, *Ctla4*, *Tnfrsf18* and *Icos*), this Treg cell program contains multiple feedback loops to reinforce expression of FOXP3 and its co-factors, such as GATA3, RUNX1 and STAT3[12-14]. This network ensures the maintenance of a stable, differentiated state observed in the Treg cell population[15] that is necessary for immune homeostasis; however, there are modifications of this state that give rise to Treg cell heterogeneity.

Of the Treg cells that are present in the circulation and lymphoid organs, most express the CC-chemokine receptor 7 (CCR7) and the adhesion receptor CD62L, which direct their recirculation through lymphoid tissues[16, 17], a population which we refer to here as the "central" Treg cell pool (**Box 1**). A minor fraction of FOXP3$^+$ Treg cells in lymphoid organs exhibit an alternative surface phenotype that is similar to activated or effector conventional T cells: CCR7^{low}CD62L^{low}CD44^{hi}KLRG1$^+$CD103$^+$. This similarity has led to their description as "effector" Treg cells, a term that is supported by the observations that this Treg cell subset is also enriched in non-lymphoid tissues and can be recruited during immune responses[17-22]. Furthermore, whole transcriptome analysis of KLRG1$^+$CD103$^+$ Treg cells from mice confirmed their distinction from central Treg cells, demonstrated their similarity to Treg cells expanded under lymphopenic conditions and revealed higher transcription of genes associated with conventional T cell activation[23]. Analogous subsets have been observed in humans, termed "resting" and "effector" Treg cells, and defined as FOXP3^{low}CD45RA^{hi}CD25^{low} and FOXP3^{hi}CD45RA^{low}CD25^{hi}, respectively[24]. As yet it is unclear whether these populations are stable, or whether individual Treg cells shift between central and effector states. Likewise, the relative contributions of thymic-derived and peripherally-induced Treg cells to each of these functional subsets is yet to be determined due to the lack of validated markers to distinguish these populations in complex contexts. *A priori*, however, there is no reason to assume the exclusion of Treg cells from either source to both the central and effector pools.

Nevertheless, the distinction between central and effector Treg cells is important because the differentiation into various effector lineages modifies the migration, homeostasis and functional capacities of Treg cells. Treg cell patrol of secondary lymphoid organs is not sufficient to maintain immunological tolerance, therefore specialisation into discrete

tissue-resident subsets is required. For instance, CD103^{+} effector Treg cells express CCR4, which is important for their recruitment to the skin and lungs[25]. Treg cells deficient for CCR4 can populate and maintain immune homeostasis in secondary lymphoid organs, such as the spleen or mesenteric lymph nodes, but their inability to traffic to the skin and lungs allows the unrestrained activation of conventional T cells at these sites, and this causes severe dermatitis and pneumonitis[25]. The concept that the Treg cell activation status and capacity to home to non-lymphoid tissues is an important aspect of their function is of relevance to any strategies that are aimed at modifying their immunosuppressive capacity.

Differentiation stimuli

What brings about this differentiation? Although many effector Treg cell characteristics are similar to antigen-experienced conventional T cells, it remains unclear whether direct TCR ligation is sufficient to drive their differentiation, or whether other stimuli (such as IL-2, inflammatory cytokines or costimulation) might be necessary *in vivo*. Antigen challenge of TCR-transgenic cells via immunisation[17,25], viral infection[26] or tetracycline-induced antigen expression in the skin[27] altered the cognate Treg cell phenotype, directed their migration into non-lymphoid tissues and their persistence as "memory" Treg cells. These studies were recently extended to show that polyclonal Treg cells responding to viral infection also differentiate into effector Treg cells and assume characteristics of memory T cells[28]. Although these findings are consistent with the view that antigen drives effector Treg cell differentiation, it will be important to test whether this is the case with other TCR specificities and to define the precise requirement for other stimuli. Observations that the provision of IL-2 *in vivo* favours the expansion of effector Treg cells[29] and that reduced IL-2R signalling in Treg cells appears to impair effector Treg cell generation[30,31] favour the view that IL-2, perhaps in conjunction with the basal TCR stimuli available to central Treg cells *in vivo*, is important. As there seem to be multiple Treg cell subsets, often associated with particular tissues (see below), it seems likely that the inductive microenvironment will play a large role in the type of effector program that is initiated.

Transcriptional control of Treg cell heterogeneity

Once engaged, the effector Treg cell program alters the basal FOXP3 transcriptional network. The pleiotropic transcription factor IRF4 seems to have a non-redundant role in effector Treg cell differentiation. Although initially described as a mediator of the differentiation of effector Treg cells that specifically antagonise T_H2 inflammatory responses in FOXP3-deficient mice[19], a recent study found evidence of broader effects of IRF4 on effector Treg cell differentiation[20]. Treg cells from *Irf4*$^{-/-}$ mice fail to downregulate CD62L and upregulate KLRG1, CTLA4, CD103, Blimp1, T-bet and ICOS, have impaired homing to non-lymphoid tissues and are unable to suppress systemic T_H1

cell responses[20,32]. Such a broad requirement resembles the role of this transcription factor in T_H17, T_H2, B cell and $CD8^+$ dendritic cell development, and is likely to involve cooperative binding with a BATF-Jun heterodimer of the activator protein 1 (AP1) transcription factor, as recently described in these other cell types[33]. Such cooperation in executing the effector Treg cell transcriptional program would enable the integration of a variety of signals to direct their suppressive function appropriately. Indeed, several different transcription factors seem capable of driving further diversification of Treg cell function on the basis of tissue localisation or the inflammatory milieu being targeted. This "polarisation" of effector Treg cells is linked to altered homing and homeostatic properties (**Figure 1**). It is also important to note that it remains unclear whether these changes in Treg cell phenotype and function represent terminal differentiation of the cells – the program may be reversible or mutable.

Do shared transcriptional modules match Treg to conventional effector cells?

A large body of recent research has focused on how effector Treg cell subsets polarise to efficiently curtail a specific T helper (T_H) cell subset. There is evidence to indicate that the transcription factors that are essential for orchestrating conventional $CD4^+$ T cell effector programs influence the polarisation of effector Treg cells. The transcription factors T-bet, GATA3, STAT3 and BCL6 have essential roles in T_H1, T_H2, T_H17 and T_{FH} cell differentiation, respectively. Treg cells also express these proteins. The conditional deletion of some of these in $FOXP3^+$ cells appears to impair their ability to antagonize the inflammation elicited by the corresponding $CD4^+$ effector T cell counterparts[34-38]. Loss of T-bet, STAT3 or BCL6 in Treg cells does not seem to affect the homeostasis of the lymphoid Treg cells, in terms of overall numbers or their capacity to compete for space with wild-type Treg cells[34,36,37]. Yet Treg cell-specific loss of these transcription factors results in a subset-specific dysregulation of T_H1, T_H17 or T_{FH} cell responses, respectively[34,36,37]. With regard to T-bet and BCL6, this function appears to be mediated at least partially through cell migration, as T-bet- and BCL6-deficient Treg cells fail to upregulate the chemokine receptors CXCR3 or CXCR5, respectively, which impairs their recruitment to sites of T_H1 or T_{FH} cell responses[34,37,39].

These studies suggest that the shared use of the transcriptional and homing machinery used by the various helper T cell effectors (in the context of the FOXP3 program) effectively matches effector Treg cells to the appropriate inflammatory milieu. This observation has prompted an attractive model that proposes that these transcriptional programs also tailor Treg cell homeostatic requirements and regulatory function to match the type of immune response driven by the various helper T cell effectors[16,32,40,41]. Indeed, the loss of STAT3 in Treg cells, for example, reduces their expression of the immunosuppressive cytokines IL-10 and IL-35[36]. However, there are notable exceptions from this apparent mirroring of transcription factor requirements in effector Treg cell

subtypes with their conventional counterparts, particularly in the control of T_H2 cell responses. As discussed above, the loss of IRF4 in Treg cells has a major impact on the entire effector Treg cell differentiation program. Furthermore, selective loss of *Gata3* in Treg cells causes general disruption of Treg cell homeostasis and leads to spontaneous autoimmune attack of the salivary glands, lacrimal glands and pancreas, with high levels of IFNγ and IL-17A found in one study[42], and impaired recruitment to inflammatory lesions in another[35]. These data indicate a much broader role for GATA3 in Treg cell function than would be predicted from the aforementioned model.

There are also other (perhaps more parsimonious) explanations of the data, which posit that the apparent adaptation of Treg cell regulatory modalities merely reflects a differential response of T_H1, T_H2, T_H17 or T_{FH} effector cell function to generic Treg cell suppressive mechanisms[43]. For example, T_H1 and T_H2 cells respond differently to changes in Treg cell number or function[44], in part due to a heightened sensitivity of T_H2 effector cells to Treg cell-derived CTLA4[43]. Further studies that distinguish effects on Treg cell homing from their various suppressive mechanisms should resolve the extent to which the classical T_H cell transcription factors shape effector Treg cell differentiation, homeostasis and suppressive mechanisms.

Tissue-resident Treg cells

In addition to their presence in the circulation and secondary lymphoid tissues, Treg cells can also be found in non-lymphoid tissue, even under non-inflammatory conditions[25]. The growing list of tissues that harbour substantial numbers of Treg cells under resting conditions includes the skin, lungs, liver, intestinal mucosa, adipose tissue and placenta[45]. In general, analysis of Treg cells resident in these tissues has revealed features of the effector Treg cell program, but with additional distinguishing features such as unique combinations of homing molecules, transcription factors, immune-regulatory mechanisms and TCR repertoire that collectively indicate substantial specialisation of Treg cells within these environments. Little is known about how tissue-resident Treg subsets alter their transcriptional programs to adapt to these very different contexts. However, recent progress in our understanding of Treg cells that reside in two important sites, the gut and adipose tissue, may provide some clues (**Figure 2**). It is worth noting that currently there are few tools available to discriminate long-term tissue-resident Treg cells from migratory effector Treg cells that transiently reside in the tissue. The relative population of these cell types within tissues thus remains unknown, and, indeed, is likely to vary between homeostatic and inflammatory conditions.

Gut Treg cells. The gastrointestinal tract harbours the largest reservoir of tissue-resident Treg cells in the body. The establishment of sufficient numbers and function of intestinal

Treg cells is essential for the maintenance of intestinal immune homeostasis, particularly in the colon[46]. Experiments with germ-free mice have shown that bacterial commensals are essential for the development of normal numbers of colonic Treg cells[47-50], with reconstitution studies showing that *Bacteriodes fragilis* and clostridial species are critical for intestinal Treg cell homeostasis[47, 48]. These findings have recently been translated with the identification of 17 clostridial strains present in human microbiota that induced the gut homing and proliferation of Treg cells[5]. Furthermore, it was found that these 17 strains provided a relatively high level of short-chain fatty acids (SCFAs)[5], which are bacterial breakdown products of plant-derived fibre. Importantly, Smith *et al*. showed that the SCFAs propionate, butyrate or acetate could restore colonic Treg cell numbers in germfree or antibiotic-treated mice and increase numbers in specific pathogen-free mice[51]. These effects were mediated in part by a receptor for SCFAs, GPR43 (encoded by *Ffar2*), which is expressed at high levels by colonic Treg cells, but not by their circulating counterparts[51]. Collectively, these studies highlight how a unique Treg cell homeostatic circuit is established by commensal/host interactions at the intestinal barrier surface to maintain immune tolerance (**Figure 2**).

Adipose tissue Treg cells. Treg cells are also highly enriched in visceral adipose tissue (VAT), accumulating to comprise more than 50% of the $CD4^+$ VAT compartment in 20wk-old mice, compared with 10-15% normally found in lymphoid tissues[52]. Comparative transcriptome analysis revealed that VAT Treg cells have key differences from their counterparts in lymphoid tissues (even effector Treg cells), including differential expression of immune-suppressive mediators (e.g. increased IL-10), chemokine receptors (such as high levels of CCR1, CCR2 and CXCR4) and transcription factors (such as high expression of GATA3 and the adipocyte regulator PPARγ)[52]. It is the high levels of the nuclear receptor PPARγ, along with FOXP3, that impart the unique features of VAT Treg cells and their accumulation at this site[53]. Perhaps the most intriguing aspect of the specialisation of VAT Treg cells to the adipose environment is the adoption of a lipid uptake pathway involving CD36[53], which suggests that the co-option of local metabolic programs may be a critical adaptation tissue-resident Treg cells (**Figure 2**).

The homeostasis of VAT Treg cells seems to be critically linked to metabolic state. Mice rendered obese by genetic or environmental means exhibit markedly lower VAT Treg cell numbers[52, 54], and this is coincident with the onset of adipose inflammation and macrophage activation[55]. Increasing or decreasing VAT Treg cell number via various means can diminish or amplify the adipose inflammation program, respectively[43, 45]. Accordingly, treatment of mice on a high-fat diet with a type 2 diabetes drug that stimulates PPARγ, Pioglitazone, could specifically restore the numbers and properties of VAT Treg cells. Interestingly, mice with a Treg cell-specific *Pparg* deletion and on a

high-fat diet showed a partial impairment in the restoration of insulin sensitivity and glucose tolerance following Pioglitazone treatment, which indicates that VAT Treg cells are an important mediator of this compound's therapeutic effect[53]. This experiment exemplifies the promise of specifically targeting the homeostatic mechanisms that govern populations of Treg cells in particular tissues or pathological situations, while sparing the central Treg cell pool.

Steady-state Treg cell homeostasis

Molecular control over circulating Treg cell number

The potent capacity of Treg cells for immune suppression depends on the maintenance of a stable population size. Perhaps surprisingly, the stability of the circulating Treg cell population is not achieved by low turnover. Rather, circulating Treg cells have a high basal proliferation rate compared with conventional T cells, with ~50% of the population undergoing division every 10 days in both mice[56] and humans[57]. Whether this high proliferation rate is driven by TCR self-reactivity or is a direct consequence of FOXP3 transcriptional program (or a combination of both factors) remains unknown. Evidence for the involvement of TCR signalling is ambiguous, with normal Treg cell homeostasis observed in ZAP70 hypomorphic mice[58], but substantial loss of Treg cells induced by the deletion of CD28 or the TCR signalling molecule LAT in mature Treg cells[59,60]. Regardless, under basal conditions the high proliferation rate of Treg cells is counterbalanced by their high apoptosis rate (as described below, this balance can be modified under none-basal conditions). The death of circulating and lymphoid organ Treg cells is induced by FOXP3-dependent phosphorylation of the pro-apoptotic protein BIM[61]. This pathway can be negated by IL-2, for which Treg cells express the high affinity receptor subunit CD25 in a DEC1/RUNX1-dependent manner[62]. IL-2 signalling upregulates the pro-survival protein MCL1[56], which antagonises the pro-apoptotic function of BIM; however, Treg cells are normally kept in a state of partial IL-2-deficiency by the FOXP3-dependent repression of autocrine[63,64] and paracrine[65,66] IL-2 production (**Figure 3**).

The unusually high turnover of the circulating Treg cell population linked to IL-2 availability allows for the rapid response to homeostatic perturbations. Following a partial loss of Treg cells, the tight regulation over IL-2 production is relaxed[56]. Conventional $CD4^+$ T cells appear to be the most important responders to this abatement of inhibition. Genetic complementation experiments have demonstrated that IL-2-deficiency in the αβ T cell lineage is sufficient to impair the Treg cell homeostasis[67], and $CD4^+$ conventional cells are the major IL-2 producers within this compartment. Furthermore, recent work suggests that ~10% of the conventional $CD4^+$ T cell pool is primed for IL-2 production[68]. This population has a transcriptional profile suggestive of partial activation and has a higher proliferative capacity when transferred into

lymphopenic hosts[68], suggesting that these primed cells have a high baseline of self-reactivity. This low level of TCR self-reactivity may be sufficient to drive IL-2 transcription but insufficient to cause proliferation, given the lower ITAM engagement threshold of cytokine production[69]. Treg cells appear to suppress IL-2 synthesis in these conventional $CD4^+$ T cells by inducing the nuclear localisation of the transcriptional repressor ICER[66]. By contrast, a reduction in Treg cell numbers de-represses IL-2 transcription in primed $CD4^+$ T cells, allowing the rapid production of IL-2. The positive feedback loop is completed by the effect of IL-2 on Treg cells. Binding to the high affinity IL-2 receptor expressed by most Treg cells suppresses their apoptosis via upregulation of MCL1 expression[56], in addition to promoting their function via mTOR activation[70, 71]. In combination with weaker costimulation-dependent effects, this feedback results in rapid expansion of Treg cells due to the increased proliferation and decreased apoptosis[56, 72]. These observations on the bulk central Treg cell population do not exclude the possibility of subsets with distinct homeostatic control mechanisms, including reliance on alternative molecular mediators or on quiescence rather than homeostatic proliferation.

Differential homeostatic mechanisms for Treg cell heterogeneity

Distinct homeostatic mechanisms regulate central and effector Treg cells. While the molecular process outlined above functions in central Treg cells and accurately describes the bulk Treg cell population in the circulation and lymphoid organs, different molecular programs are initiated in effector and polarised Treg cells in the basal state. The transcription factor Blimp1 seems to have a critical role in restraining effector Treg cell numbers in non-lymphoid tissues and germinal centre reactions[37, 73]. Although Blimp1 is downstream of IRF4 and is not required for effector Treg cell generation *per se*, Blimp1 directs a transcriptional program in this subset that is required for optimal IL-10 and ICOS expression and causes the downregulation of the pro-survival protein BCL2. This BCL2 downregulation may account for the 2-10-fold higher accumulation of Blimp1-deficient effector Treg cells compared to wild-type competitors in germinal centres[37], the colon and the lungs[73], a phenomenon which may have broader tissue-specificity but is yet to be systematically assessed. These findings suggest that effector Treg cells use a pro-survival program that is distinct from their central counterparts.

There may also be tissue- or inflammation-specific homeostatic cues that control effector Treg cell populations. T-bet expression in Treg cells was found to be important for their competitive fitness by enabling their proliferation in T_H1-driven inflammatory conditions[34]. Furthermore, T-bet-deficient Treg cells were comprehensively outcompeted by wild-type counterparts in all sites during the T_H1 cell response elicited by *Mycobacterium tuberculosis* infection. One specific molecular mechanism for inflammatory-specific

homeostatic cues is the interaction between NRP1, which is expressed by Treg cells, and SEMA4a, which is expressed by inflammatory tissues[74]. Although dispensable for Treg cell homeostasis in circulation or secondary lymphoid organs, the engagement of this receptor-ligand pair in inflamed tissue upregulates expression of the pro-survival protein BCL2 and thereby decreases apoptosis[74], presumably at least partially substituting for the IL-2-MCL1 axis. It will be interesting to see whether other distinct homeostatic controls are imposed in Treg cells recruited under different contexts — a theme that is emerging for the maintenance of Treg cells that reside in non-lymphoid tissues in the steady-state.

Treg cell homeostatic networks

The molecular basis of Treg cell homeostasis results in three highly important emergent properties, namely: i) rapid self-correction; ii) a homeostatic set-point based on function, and iii) competition effects.

Rapid self-correction. The first key property, that of rapid self-correction, is due to the niche-filling response being based on primed properties. The Treg cell population is poised to make rapid changes in its size because of its extremely high turnover. Slight adjustments of the proliferative or apoptotic rates of Treg cells have a large impact on their cell number, such as the expansion that occurs when IL-2-mediated increases in MCL1 expression antagonise the apoptotic machinery[56]. Likewise, the self-reactive subset of conventional $CD4^+$ T cells that first respond to Treg cell deficiency are primed for IL-2 expression[68]. The evolution of highly sensitive Treg cell responses to IL-2 (perhaps one of the earliest signs of $CD4^+$ T cell activation, occurring before proliferation or inflammatory cytokine production) has allowed the niche-sensing and response mechanism to be initiated earlier than the pathological consequences of Treg cell insufficiency.

Homeostatic set-point based on function. The second key property of Treg cell homeostasis is the dependence of feedback on their net functional capacity, rather than number, of Treg cells. By comparison, homeostasis of conventional T cells is based on IL-7 consumption[75]. Since stromal cells constitutively produce IL-7[76], this homeostatic system is non-responsive to T cell function, as non-functional or partially functional T cells can still occupy the conventional T cell niche as long as they are able to respond to IL-7. By contrast, IL-2 production is inversely correlated with the functional capacity of Treg cells to suppress conventional T cells[68]. The effect of IL-2 on the Treg cell population (outlined above) should theoretically allow compensation of minor variations in Treg cell function with complementary adjustments in Treg cell number. Conceptually similar feedback loops controlling Treg cell function based on CTLA4 modulation of co-stimulation[77] combine to generate a very robust level of net suppressive capacity, which can compensate for multiple genetic or environmental insults.

Competition effects. The third key property of Treg cell homeostasis is that the IL-2 dependence of the central Treg cell pool makes homeostasis susceptible to IL-2 competition. In the absence of immune stimulation, Treg cells constitute the vast majority of cells expressing the high affinity receptor for IL-2, providing a competitive advantage over conventional T cells expressing only the low affinity receptor complex. During infection, the transient upregulation of CD25 by effector T cells allows the clonal expansion of activated T cells that consume IL-2. This effect both directly drives the expansion of activated T cells, and also temporarily forces Treg cell contraction by depriving them of IL-2[78] (a reciprocal interaction to the proposed IL-2 consumption model for Treg cell suppression). The lag-time between immune activation and the expansion of Treg cell numbers to suppress the response may therefore depend on the kinetics of IL-2 production and the duration of CD25 expression on effector lineages. This interplay may be pertinent in several cases of immune pathology associated with altered Treg cell homeostasis[79] (see below).

In addition to competition for IL-2 from conventional T cells, the central Treg cell pool may compete with the various effector Treg cell subsets, including tissue-infiltrating T cells. Although the homeostatic mechanisms of effector Treg cell subsets are poorly characterised, at least some of these subsets are maintained independently of IL-2. For example, the homeostatic control of mucosal Treg cells is dictated by metabolites from commensal microflora[51]. Likewise, antigen-experienced memory Treg cells in the skin depend on IL-7 rather than IL-2 for homeostatic maintenance[80]. Another subset of memory Treg cells, which is still poorly described, is largely IL-15 dependent and accumulates with age[81]. Despite several of these subsets being IL-2 independent, they can express comparable CD25 levels to the undifferentiated Treg cell pool[80], and thus in principle should be able to favourably compete for IL-2, in effect siphoning IL-2 away from the central Treg cell pool. An advantage to this one-way competition effect is that it would allow the directed expansion of effector Treg cell pools at the expense of contributions from the central Treg cell pool, which supports a more specialised Treg cell response without raising the overall burden of immune suppression (**Figure 4**). Likewise, the substitution of IL-2-dependent MCL1 regulation for NRP1-dependent BCL2 regulation at inflammatory sites probably serves to allow local Treg cell populations to expand in inflamed tissues without competition from central Treg cells[74].

Disease and the dysregulation of Treg cell homeostasis

Genetic defects in Treg cell homeostasis

The dynamic nature of Treg cell homeostasis creates a resilient steady-state of Treg cell-mediated suppression. Nevertheless, there is growing evidence that defects in their homeostatic mechanisms, of either genetic or environmental origin, can drive the immune

system towards pathology. It is clear that circulating Treg cell homeostasis can be rendered dysfunctional via Mendelian defects in the IL-2 pathway. Mice with deletion of the genes encoding IL-2[82-84], IL-2Rα (CD25)[85], IL-2Rβ[86, 87] or the downstream signalling mediator Stat5[88, 89] all have impaired Treg cell homeostasis and develop fatal autoimmunity. Similar phenotypes are observed in human patients with severe loss of function mutations in *IL2RA*[90, 91] and *STAT5B*[92], although the *STAT5B* mutation-induced phenotype is complicated by the additional role of STAT5 in growth factor signalling. More commonly, hypomorphic alleles may act in a polygenic manner to increase susceptibility to autoimmunity. For instance, the *Idd3* polymorphism in NOD mice results in lower IL-2 production by conventional T cells and a lower proportion of Treg cells with impaired suppressive function, and this significantly contributes to the susceptibility of this strain to autoimmunity[93]. In humans, the prime example is polymorphisms in *IL2RA*, which are strongly associated with several autoimmune diseases, including type 1 diabetes and multiple sclerosis[94, 95].

Treg cell homeostasis defects during graft versus host disease

In addition to Treg cell homeostatic defects created by impairment of signalling between effector T cells and Treg cells, Treg cell homeostasis can be distorted by the loss of effector T cells, indirectly reducing support of the Treg cell niche. Possibly the best described condition of this type is graft versus host disease (GvHD). Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is a common treatment for many haematological malignancies. To accelerate immune recovery, protect patients from infections and generate a graft versus tumour effect, donor T cells are usually transferred together with the haematopoietic stem cells[96]. However, this strategy limits the success of allo-HSCT by facilitating the development of GvHD. GvHD is caused by the donor T cell response to the recipient's organs and tissues (particularly the skin, liver and gut). Disease can be discriminated between acute GvHD (onset before 100 days after transplantation) and chronic GvHD (after 100 days), which involve different inflammatory components[96]. There is strong evidence to suggest that acute GvHD is a disease of Treg cell homeostasis, caused by an insufficient population of IL-2-producing conventional T cells. In mouse models, the adoptive transfer of Treg cells or recombinant IL-2 delays acute GvHD onset and improves immune recovery[97-101]. In patients, the incidence of GvHD negatively correlates with the frequency of donor Treg cells in peripheral blood of HSCT recipients[102-106]. Several phase I clinical trials designed to test the safety of exogenous Treg cell supplementation after allogenic HSCT started with very promising results in the prevention of GvHD[107, 108]. However, a major limitation in these trials was the rapid loss of transferred Treg cells[107], supporting the supposition that the Treg cell niche is restricted due to limiting numbers conventional T cells and thus inadequate IL-2 production. Likewise, direct IL-2 supplementation can temporarily boost Treg cell numbers and prevent GvHD[109, 110]; however, until endogenous IL-2-producing

conventional T cells recover, continual IL-2 supplementation is required to maintain Treg cell numbers.

Infection-induced defects in Treg cell homeostatic correction

Another avenue for disease to develop from disturbed Treg cell homeostasis is through perturbations of the IL-2-dependent feedback loop by infection. In the homeostatic context Treg cells are major consumers of IL-2; indeed competitive consumption of IL-2 is proposed to be one of the functional mediators of Treg cell suppression, as it limits IL-2 available for both conventional T cells[111] and NK cells[112]. During a normal infection, IL-2 production correlates with inflammatory cytokine production[113], allowing Treg cell numbers to track with inflammation. This process limits immune pathology[113] and serves to shutdown the immune response once effector cells subside (**Figure 5A,B**). However, in exceptional cases the direct relationship between inflammatory cytokines and IL-2 production can break down. The prime example of this is *Toxoplasma gondii* infection, which can lead to lethal immune pathology[114]. During a fatal *T. gondii* infection, excessive IFNγ production shuts down IL-2 production, triggering a homeostatic Treg cell contraction and allowing the development of immune pathology[79]. A similar effect can be initiated by IL-27 production by inflammatory cells, which reduces IL-2 and hence the Treg cell population, leading to autoinflammatory disease[115]. It is also notable that the correlation between effector function and IL-2 production breaks down with T_H17 cell responses, as Aiolos expression by T_H17 cells silences IL-2 production[116]. This mechanism is thought to make T_H17 cell responses more self-limiting compared with other T_H cell responses, but it may also prevent efficient Treg cell homeostatic responses to strong T_H17 cell responses, which explains the dominant association of T_H17 cells with autoimmunity. An additional mechanism by which the relationship between inflammation and IL-2 availability can break down is through the consumption of IL-2 by non-regulatory T cells. Indeed, activated effector CD4 T cells during infection with *T. gondii*, *Listeria monocytogenes* and vaccine virus consume sufficient IL-2 to reduce the amount available to Treg cells below homeostatic requirements[78], a process which may contribute to immune pathology during these severe infections. Likewise, evidence suggests that following excessive activation, $CD8^+$ T cells can be induced to express CD25 to levels such that IL-2 consumption by $CD8^+$ T cells may reduce IL-2 availability to levels too low to maintain Treg cell numbers, resulting in immune pathology (S. Humblet-Baron, personal communication). In all of these cases, the normal homeostatic correction mechanisms fail, such that there is insufficient IL-2 to support Treg cell expansion. In severe cases, this may lead to a positive inflammatory feedback loop that supports autoinflammation (**Figure 5C**).

Finally, there is growing evidence that septic shock syndrome involves defective Treg cell homeostasis. Sepsis and septic shock are life-threatening complications of infection.

It has become clear that the initial response to septic shock is a hyperinflammatory reaction, typically mediated by endotoxin, releasing a plethora of pro-inflammatory cytokines and other mediators into the circulation. This acute stage is accompanied by a deficit in Treg cells, which may contribute to the hyperinflammatory state[117]. Most patients can survive this acute stage with best medical practice of pulmonary and cardiovascular support in an intensive care unit. However, patients surviving this activation phase often become immune suppressed, such that the original infection is unresolved and the patient is at risk of additional opportunistic infections. This immune paralysis has been found to be associated with a reduction of circulating effector cells with curtailed potential to produce pro-inflammatory cytokines[117]. The excessive expansion of Treg cells in this latter stage of disease is likely to be an important mechanism that induces this lymphocytic suppression[118, 119]. Indeed, removal of surplus Treg cells in both mice and human patients seems to mitigate immunosuppression[119, 120]. This "over-filling" of the Treg cell niche is reminiscent of the excessive expansion that is observed in a partial depletion model in mice[56]. It is therefore worth speculating that septic shock syndrome consists of dual modalities of Treg cell homeostatic failure: a reduction of Treg cells during the acute phase, leading to hyperinflammation, followed by homeostatic expansion "over-filling" of Treg cells, leading to an anergic phase.

Perspectives

The past year has provided striking new insights into the molecular machinery that controls Treg cell homeostasis and their differentiation into diverse functional subsets. The basal FOXP3-dependent transcriptional network engaged by central Treg cells induces a program of high turnover, primed to react to minor changes in conventional T cell activation status, primarily sensed via IL-2. These properties allow for the rapid responses that are required from the Treg cell population at the earliest signs of reactivity, due to dynamic nature of immunity and inflammation. The importance of these properties is emphasised by the devastating immune conditions that are caused by genetic or environmental disruption of Treg cell homeostatic networks.

Another important theme that has emerged is the specialisation of Treg cells for tissue- and inflammation-specific responses. At the transcriptional level, many familiar players appear to act in concert with the FOXP3-induced program to drive Treg cell diversification. Tailoring effector Treg cell proliferative and apoptotic controls to specific inflammatory milieu would promote immune balance, allowing for appropriate antagonism of inflammation during expansion, followed by relief of immunosuppression during contraction. The contraction phase may prove to be important for processes such as the generation of immunological memory or healing in non-lymphoid tissues. Regardless, there is a pressing need to better define the homeostatic mechanisms that

control central, effector and polarised Treg cell populations, given the obvious potential for the targeted modulation of Treg cells populations pertinent to pathology.

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Competing Financial Interests

The authors declare no competing financial interests.

Call-out material

Box 1. Treg cells nomenclature

Foxp3$^+$ Treg cells are heterogeneous, with multiple possible origins (thymic and peripheral induction) and various peripheral functional profiles. Both the nomenclature and defining markers of these profiles remains non-standardized. In this review we have grouped the peripheral Treg cell pool into three classifications:

"Central" Treg cells. Central Treg cells are the majority of Treg cells in circulation and in secondary lymphoid organs. Also called "resting" or "naïve" Treg cells in some studies, this population shares phenotypic features with naïve and memory conventional T cells (including activation markers and circulation patterns), yet they are not quiescent, with baseline suppressive function and a history of antigen exposure. Studies reviewed here that used CD62L^{hi}, CCR7$^+$CD62L$^+$ or CD45RA^{hi}CD25^{low} Treg cells are considered to reflect this central Treg cell population, as are studies performed on bulk Treg cells collected from the secondary lymphoid organs, due to the preponderance of this population.

"Effector" Treg cells. Effector Treg cells are the minor fraction of Treg cells in circulation and in secondary lymphoid organs, and are called "activated" Treg cells in some studies. This population shares phenotypic features with activated conventional T cells, and are variously defined as CD62L^{low}, CCR7^{low}CD62L^{low}CD44^{hi}KLRG1$^+$CD103$^+$, or CD45RA^{low}CD25^{hi}, depending on the study. Treg cells with this profile are thought to have undergone more recent antigen encounter than central Treg cells, and show enhanced migration through non-lymphoid tissues. It is unclear whether effector Treg cells are terminally differentiated or whether they are capable of returning into a central Treg cell state.

"Tissue-resident" Treg cells. Tissue-resident Treg cells are those Treg cells that have long-term residence in non-lymphoid tissues, as opposed to the short-term migration through non-lymphoid tissues observed by effector Treg cells. Potentially, each organ may harbor a distinct population of tissue-resident Treg cells, which may adopt functions in addition to local immune regulation. Currently the best examples of tissue-resident Treg cells are adipose tissue Treg cells, marked by PPARγ expression, and gut-resident Treg cells, marked by GPR43.

Figures

Figure 1. Treg cell activation and differentiation. FOXP3+ Treg cells are exported from the thymus and recirculate through secondary lymphoid tissues as central Treg cells. Activation signals involving TCR ligation, CD28 costimulation and/or IL-2 induce the upregulation of IRF4, which orchestrates their differentiation into effector Treg cells. Further effector Treg cell differentiation involves BACH2 downregulation and Blimp1 upregulation. Unknown stimuli induce the polarisation of Treg cells by upregulating transcription factors that can act together with FOXP3 to induce the expression of chemokine and homing receptors that mediate their recruitment to tissues or sites of inflammation. Various immune or tissue-specific mediators are important for Treg cell homeostasis or suppressive function at these sites. Dashed lines indicate uncertainty over the reversibility of differentiation. SCFAs, short-chain fatty acids; LCFAs, long-chain fatty acids.

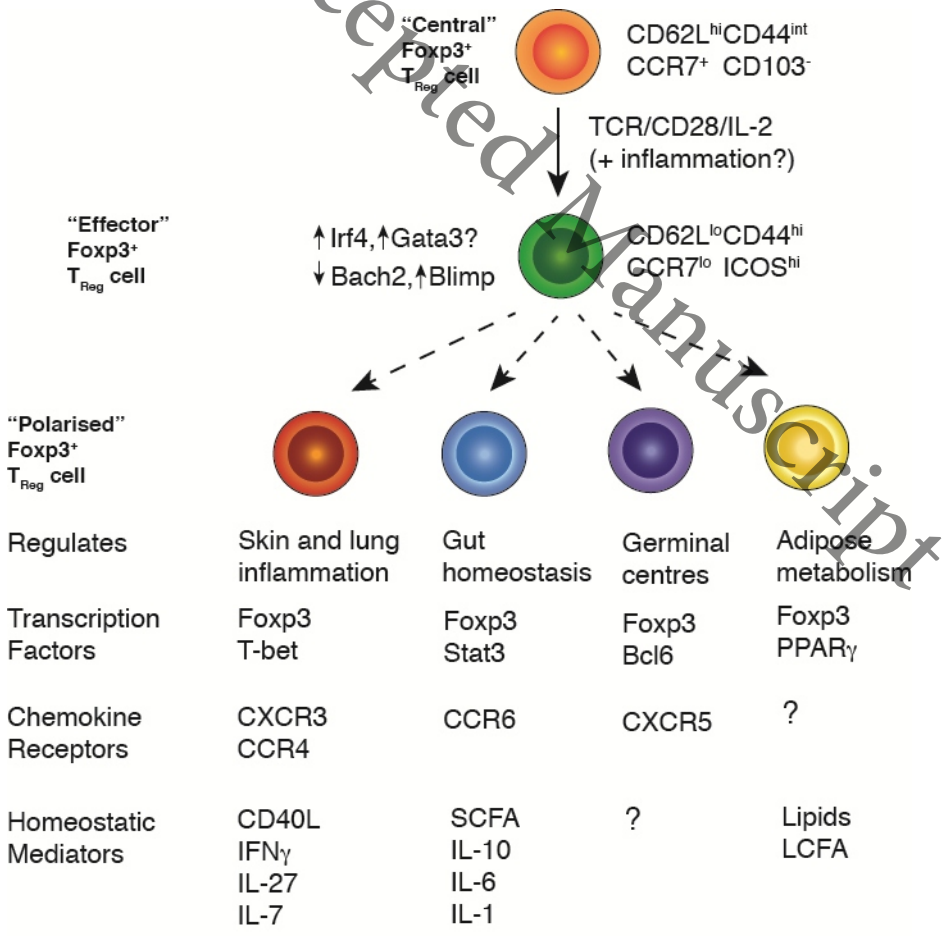


Figure 2. Molecular mediators of tissue-resident Treg cell homeostasis. Examples of the diverse stimuli integrated by tissue-resident Treg cells to maintain their survival programs within specialised microenvironments. Adipose or gut tissue Treg cells express restricted TCR repertoires that may recognise adipose- or gut-/commensal-specific antigens, respectively. A unique chemokine and adhesion receptor combination recruits and confines cells to their respective tissues, while receptors such as CD36 or GPR43 sense environment-specific factors, including lipids or microbial short-chain fatty acids. Integration of these signals with those from other receptors specific for tissue-resident Treg cells, or expressed by all Treg cells (such as IL-2R), culminate in a survival program required to maintain Treg cell specialisation.

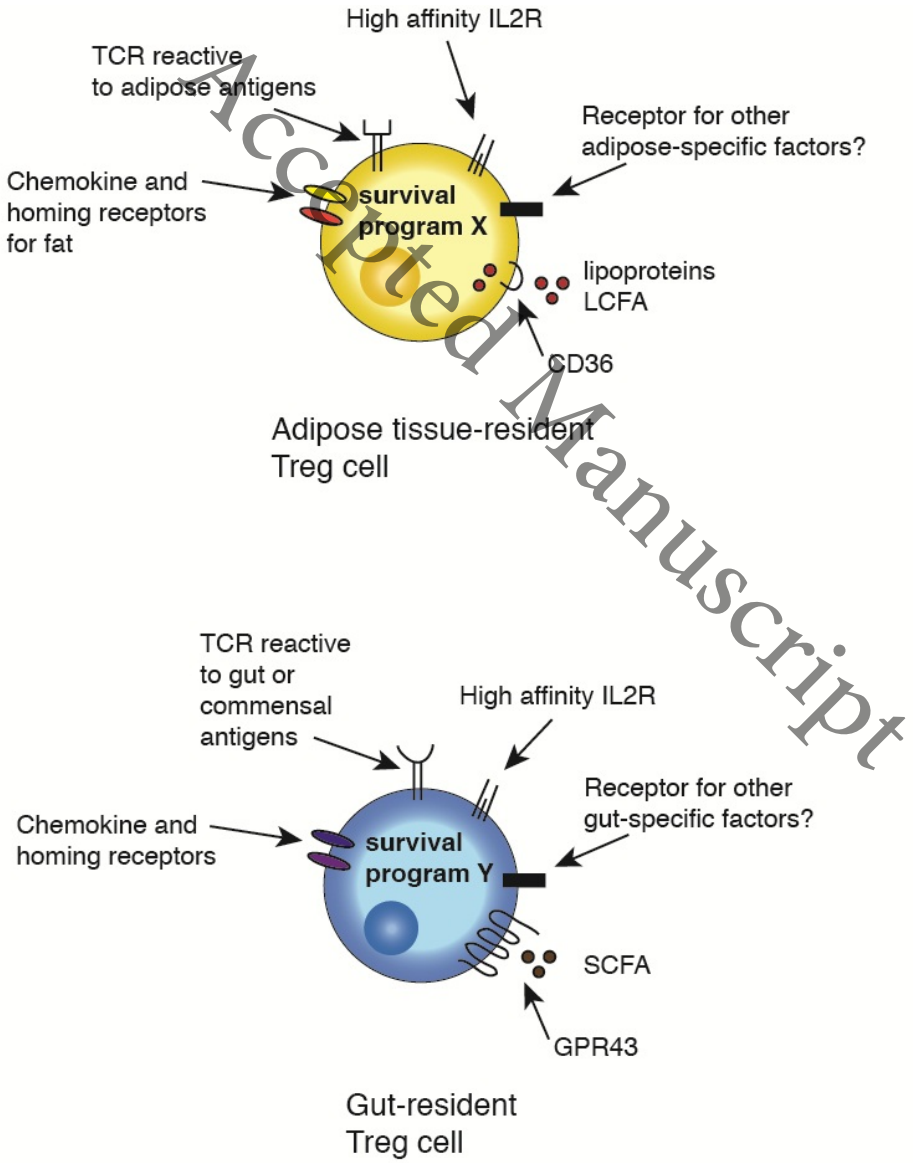


Figure 3. Molecular control over Treg cell homeostasis. A. Under steady state conditions, the apoptotic balance is maintained by the pro-survival factor MCL1 and the pro-apoptotic protein BIM, feeding through to the intrinsic apoptotic mediators BAK and BAX. **B.** Under conditions of central Treg cell deficiency, additional IL-2 is produced from activated conventional T cells, leading to a shift in the MCL1:BIM ratio to a pro-survival balance, allowing Treg cell expansion. **C.** Under conditions of central Treg cell surplus, efficient quenching of IL-2 production by conventional T cells leads to a relative IL-2-deficiency that shifts the MCL1:BIM ratio to a pro-apoptotic balance, driving Treg cell contraction.

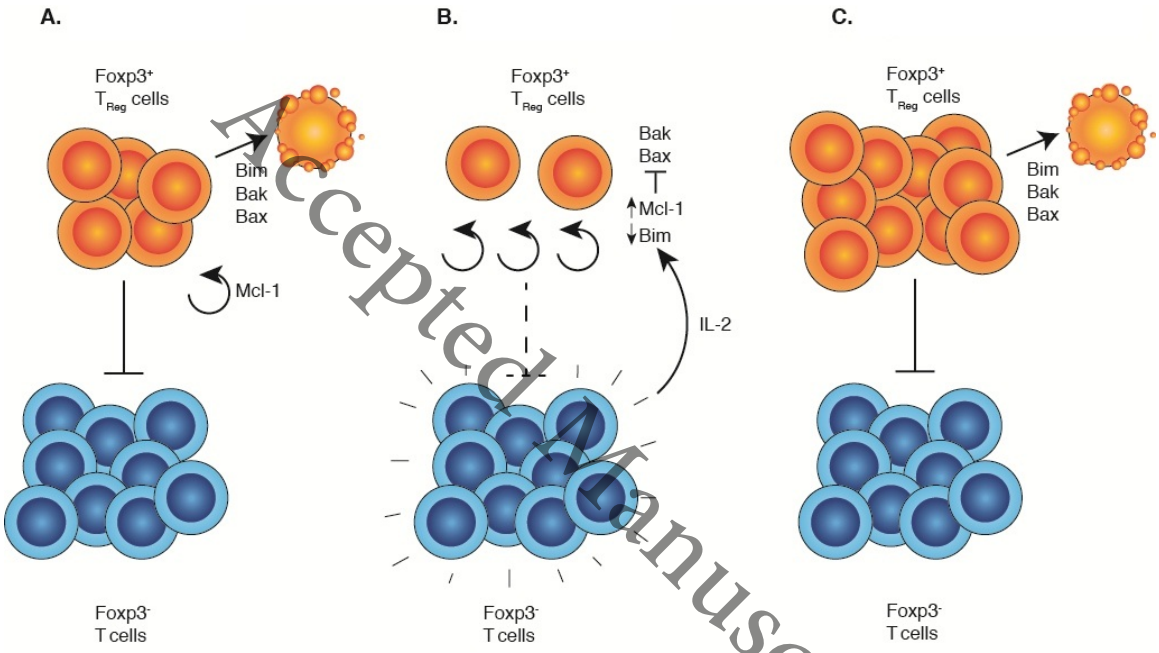


Figure 4. Competitive and non-competitive models for interactions between Treg cell subsets. The differentiation of the Treg cell pool into multiple subsets with distinct homeostatic properties requires the development of a complex model for Treg cell homeostasis that takes into account potential subset interaction. **A.** In the non-competitive model, the generation of new Treg cell subsets with distinct homeostatic properties has no impact on the undifferentiated Treg cell pool. Thus the addition of a colonic Treg cell subset via microbial colonisation (blue) or a skin memory Treg cell subset via antigen exposure in the tissue (green) increases the size of the total Treg cell pool and thus the regulatory burden on the immune system in a directly additive manner. **B.** In a strictly competitive model, the generation of new Treg cell subsets with distinct homeostatic control competes directly with the undifferentiated Treg cell pool for IL-2 availability, even though the differentiated subsets are IL-2-independent. In this scenario the total Treg cell pool becomes specialised without increasing the regulatory burden on the immune system. **C.** In a mixed model, there will be Treg cell subsets that are IL-2-independent and low IL-2 consumers (eg, colonic Treg cells) and Treg cell subsets that are IL-2-independent and high IL-2 consumers (eg, skin memory Treg cells), allowing increased diversity of the total Treg cell pool while limiting the increase in regulatory burden.

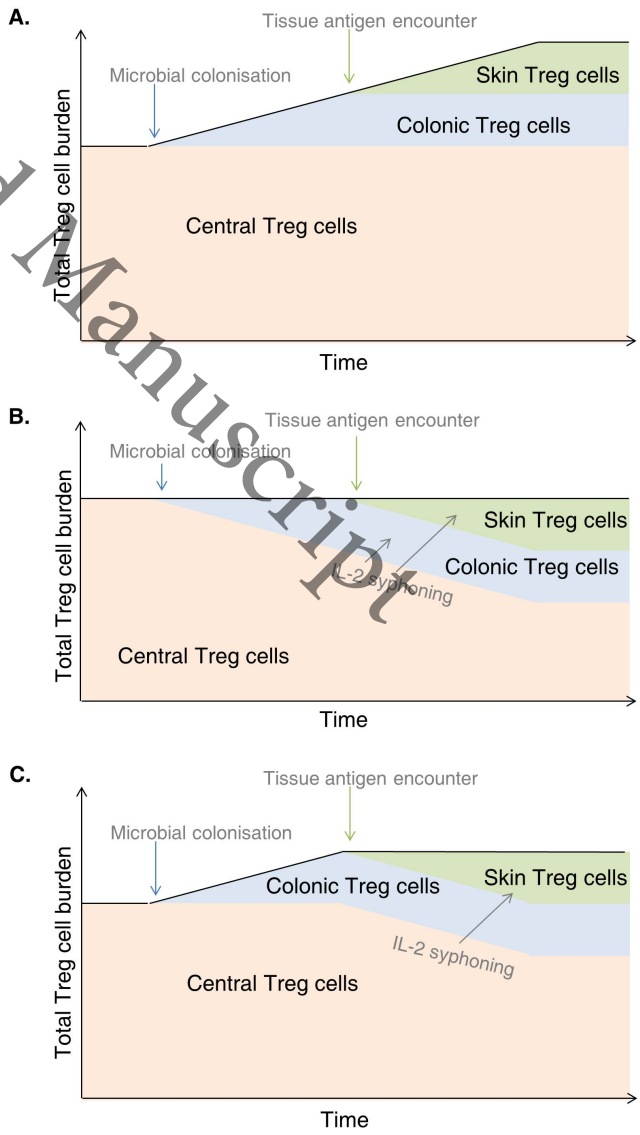
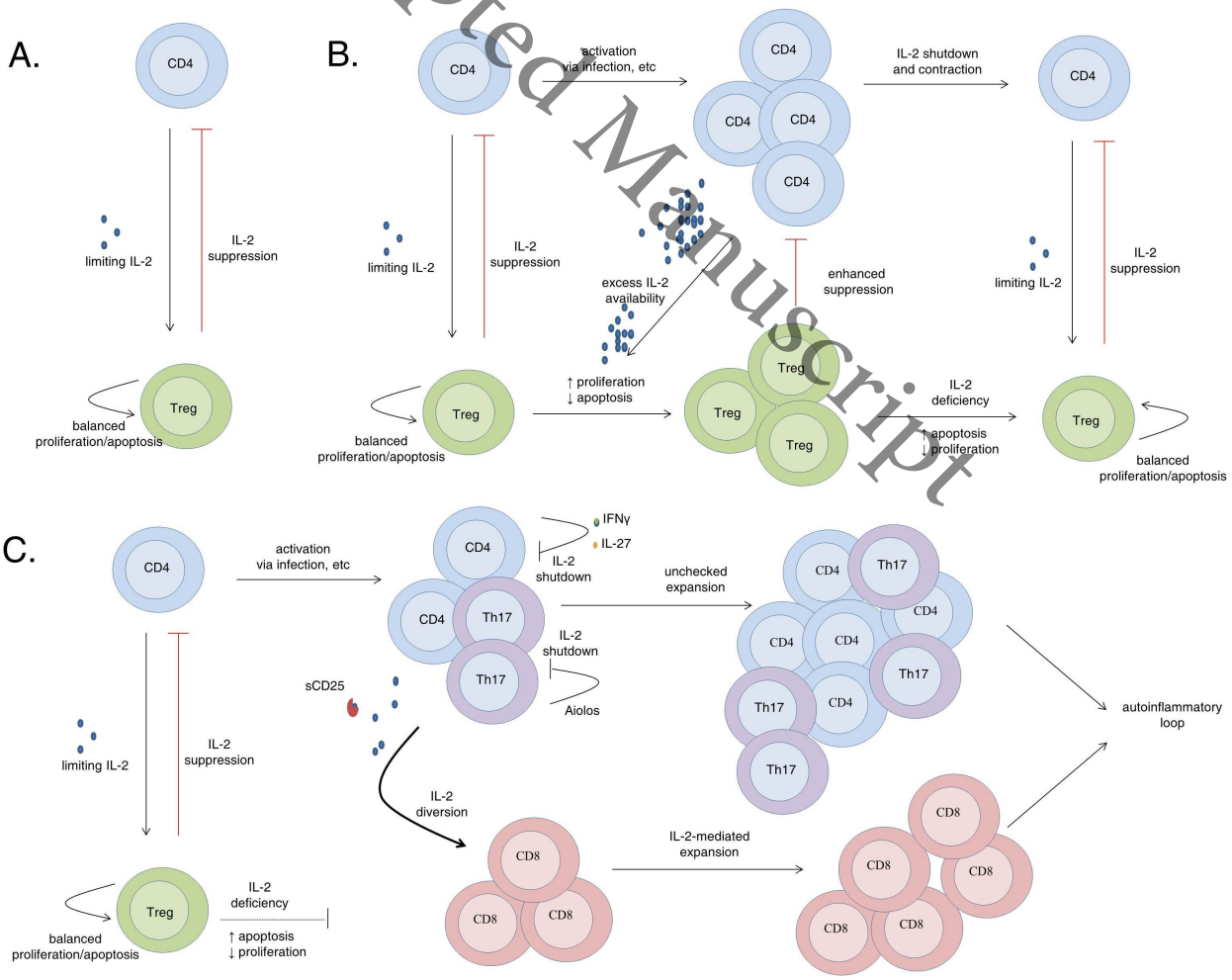


Figure 5. Models for Treg cell homeostasis disorders triggered by infection. A. In the normal homeostatic state, a low activity balance is generated by limiting IL-2 production from conventional $CD4^+$ T cells. This partial IL-2 deficiency results in balanced proliferation/apoptosis in Treg cells, which in turn keep IL-2 production by $CD4^+$ T cells low. **B.** This state of low activity balance can be temporarily suspended by an infection, resulting in $CD4^+$ T cell expansion and activation. One component of this activation is the production of excess IL-2, which allows Treg cell expansion. As a consequence of Treg cell expansion, the enhanced suppressive capacities shut down the $CD4^+$ T cell response until IL-2 becomes limiting. Finally, Treg cells contract via IL-2-deficiency and the system returns to homeostasis. **C.** This normal process can be derailed through several processes. Firstly, if the immune response is primarily T_H17 type in nature, expression of Aiolos inhibits production of IL-2. Secondly, strong inflammatory production of IFNγ or IL-27 can reduce IL-2 production. Third, shedding of CD25 into the soluble CD25 isoform may sequester bioactive IL-2. Fourth, activated $CD8^+$ T cells may effectively compete for available IL-2 due to high CD25 expression. Under these conditions, Treg cells may experience severe IL-2 deficiency and contract rather than expand. The loss of suppressive function may then spur further inflammatory activation and lead to an autoinflammatory feedback loop.



Glossary

Graft versus tumour effect

The antitumour activity of donor T cells against residual malignant cells of the graft recipient following (allogeneic) bone marrow transplantation.

PPARγ

A member of a group of nuclear receptor proteins involved in altering lipid and glucose metabolism. Their ligands include free fatty acids and eicosanoids.

Recent thymic emigrant

A semi-mature T cell that has left the thymus but has yet to undergo the final stages of maturation. Typically a window of around two weeks post-thymic maturation is used to differentiate between recent thymic emigrants and fully mature T cells.

Specific pathogen-free mice

Mice kept in specific vivarium conditions whereby a number of pathogens, such as most of the known chronic and latent persistent pathogens, are excluded or eradicated from the colony. Although this enables better control of experimental conditions related to immunity and infection, it also sets apart such animal models from pathogen-exposed humans or non-human primates, whose immune systems are in constant contact with potential pathogens.

Visceral adipose tissue

Visceral or abdominal fat is located in between the peritoneal organs and is distinct from subcutaneous and intramuscular fat.

References

1. Liston, A. & Piccirillo, C. A. Developmental plasticity of murine and human Foxp3(+) regulatory T cells. *Advances in immunology* **119**, 85-106 (2013).
2. Hsieh, C. S., Lee, H. M. & Lio, C. W. Selection of regulatory T cells in the thymus. *Nat Rev Immunol* **12**, 157-167 (2012).
3. Paiva, R. S. *et al*. Recent thymic emigrants are the preferential precursors of regulatory T cells differentiated in the periphery. *Proc Natl Acad Sci U S A* **110**, 6494-6499 (2013).
4. Mabarrack, N. H., Turner, N. L. & Mayrhofer, G. Recent thymic origin, differentiation, and turnover of regulatory T cells. *Journal of leukocyte biology* **84**, 1287-1297 (2008).
5. Atarashi, K. *et al*. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* **500**, 232-236 (2013).
6. Samstein, R. M. *et al*. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* **150**, 29-38 (2012).
7. Josefowicz, S. Z. *et al*. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* **482**, 395-399 (2012).

8. Thornton, A. M. *et al*. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* **184**, 3433-3441 (2010).
9. Weiss, J. M. *et al*. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* **209**, 1723-1742, S1721 (2012).
10. Yadav, M. *et al*. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med* **209**, 1713-1722, S1711-1719 (2012).
11. Williams, L. M. & Rudensky, A. Y. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* **8**, 277-284 (2007).
12. Fu, W. *et al*. A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells. *Nat Immunol* **13**, 972-980 (2012).
13. Rudra, D. *et al*. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol* **13**, 1010-1019 (2012).
14. Kitoh, A. *et al*. Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells. *Immunity* **31**, 609-620 (2009).
15. Rubtsov, Y. P. *et al*. Stability of the regulatory T cell lineage in vivo. *Science* **329**, 1667-1671 (2010).
16. Campbell, D. J. & Koch, M. A. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat Rev Immunol* **11**, 119-130 (2011).
17. Lee, J. H., Kang, S. G. & Kim, C. H. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol* **178**, 301-311 (2007).
18. Kleinewietfeld, M. *et al*. CCR6 expression defines regulatory effector/memory-like cells within the CD25(+)CD4+ T-cell subset. *Blood* **105**, 2877-2886 (2005).
19. Huehn, J. *et al*. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med* **199**, 303-313 (2004).
20. Suffia, I., Reckling, S. K., Salay, G. & Belkaid, Y. A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. *J Immunol* **174**, 5444-5455 (2005).
21. Stephens, G. L., Andersson, J. & Shevach, E. M. Distinct subsets of FoxP3+ regulatory T cells participate in the control of immune responses. *J Immunol* **178**, 6901-6911 (2007).
22. Beyersdorf, N., Ding, X., Tietze, J. K. & Hanke, T. Characterization of mouse CD4 T cell subsets defined by expression of KLRG1. *Eur J Immunol* **37**, 3445-3454 (2007).
23. Feuerer, M. *et al*. Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A* **107**, 5919-5924 (2010).
24. Miyara, M. *et al*. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* **30**, 899-911 (2009).

25. Sather, B. D. *et al*. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med* **204**, 1335-1347 (2007).
26. Sanchez, A. M., Zhu, J., Huang, X. & Yang, Y. The development and function of memory regulatory T cells after acute viral infections. *J Immunol* **189**, 2805-2814 (2012).
27. Rosenblum, M. D. *et al*. Response to self antigen imprints regulatory memory in tissues. *Nature* **480**, 538-542 (2011).
28. Brincks, E. L. *et al*. Antigen-specific memory regulatory CD4+Foxp3+ T cells control memory responses to influenza virus infection. *J Immunol* **190**, 3438-3446 (2013).
29. Webster, K. E. *et al*. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J Exp Med* **206**, 751-760 (2009).
30. Cheng, G., Yu, A., Dee, M. J. & Malek, T. R. IL-2R signaling is essential for functional maturation of regulatory T cells during thymic development. *J Immunol* **190**, 1567-1575 (2013).
31. Yu, A., Zhu, L., Altman, N. H. & Malek, T. R. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity* **30**, 204-217 (2009).
32. Cretney, E., Kallies, A. & Nutt, S. L. Differentiation and function of Foxp3(+) effector regulatory T cells. *Trends in immunology* **34**, 74-80 (2013).
33. Martinez, G. J. & Rao, A. Immunology. Cooperative transcription factor complexes in control. *Science* **338**, 891-892 (2012).
34. Koch, M. A. *et al*. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* **10**, 595-602 (2009).
35. Wohlfert, E. A. *et al*. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest* **121**, 4503-4515 (2011).
36. Chaudhry, A. *et al*. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* **326**, 986-991 (2009).
37. Linterman, M. A. *et al*. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* **17**, 975-982 (2011).
38. Chung, Y. *et al*. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* **17**, 983-988 (2011).
39. Wollenberg, I. *et al*. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* **187**, 4553-4560 (2011).
40. Chaudhry, A. & Rudensky, A. Y. Control of inflammation by integration of environmental cues by regulatory T cells. *J Clin Invest* **123**, 939-944 (2013).
41. Josefowicz, S. Z., Lu, L. F. & Rudensky, A. Y. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annu Rev Immunol*(2012).
42. Wang, Y., Su, M. A. & Wan, Y. Y. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity* **35**, 337-348 (2011).
43. Tian, L., Humblet-Baron, S. & Liston, A. Immune tolerance: are regulatory T cell subsets needed to explain suppression of autoimmunity? *BioEssays : news and reviews in molecular, cellular and developmental biology* **34**, 569-575 (2012).

44. Tian, L. *et al*. Foxp3(+) regulatory T cells exert asymmetric control over murine helper responses by inducing Th2 cell apoptosis. *Blood* **118**, 1845-1853 (2011).
45. Burzyn, D., Benoist, C. & Mathis, D. Regulatory T cells in nonlymphoid tissues. *Nat Immunol* **14**, 1007-1013 (2013).
46. Littman, D. R. & Rudensky, A. Y. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* **140**, 845-858 (2010).
47. Round, J. L. & Mazmanian, S. K. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* **107**, 12204-12209 (2010).
48. Atarashi, K. *et al*. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* **331**, 337-341 (2011).
49. Geuking, M. B. *et al*. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* **34**, 794-806 (2011).
50. Russell, S. L. *et al*. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO reports* **13**, 440-447 (2012).
51. Smith, P. M. *et al*. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569-573 (2013).
52. Feuerer, M. *et al*. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* **15**, 930-939 (2009).
53. Cipolletta, D. *et al*. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* **486**, 549-553 (2012).
54. Deiuliis, J. *et al*. Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers. *PLoS One* **6**, e16376 (2011).
55. Shoelson, S. E., Lee, J. & Goldfine, A. B. Inflammation and insulin resistance. *J Clin Invest* **116**, 1793-1801 (2006).
56. Pierson, W. *et al*. Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3 regulatory T cells. *Nat Immunol*(2013).
57. Vukmanovic-Stejic, M. *et al*. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* **116**, 2423-2433 (2006).
58. Siggs, O. M. *et al*. Opposing functions of the T cell receptor kinase ZAP-70 in immunity and tolerance differentially titrate in response to nucleotide substitutions. *Immunity* **27**, 912-926 (2007).
59. Shen, S. *et al*. The importance of LAT in the activation, homeostasis, and regulatory function of T cells. *J Biol Chem* **285**, 35393-35405 (2010).
60. Gogishvili, T. *et al*. Cell-intrinsic and -extrinsic control of Treg-cell homeostasis and function revealed by induced CD28 deletion. *Eur J Immunol* **43**, 188-193 (2013).
61. Tai, X. *et al*. Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals. *Immunity* **38**, 1116-1128 (2013).
62. Miyazaki, K. *et al*. The role of the basic helix-loop-helix transcription factor Dec1 in the regulatory T cells. *J Immunol* **185**, 7330-7339 (2010).
63. Ono, M. *et al*. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* **446**, 685-689 (2007).

64. Wu, Y. *et al*. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* **126**, 375-387 (2006).
65. Thornton, A. M. & Shevach, E. M. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* **188**, 287-296 (1998).
66. Vaeth, M. *et al*. Regulatory T cells facilitate the nuclear accumulation of inducible cAMP early repressor (ICER) and suppress nuclear factor of activated T cell c1 (NFATc1). *Proc Natl Acad Sci U S A* **108**, 2480-2485 (2011).
67. Almeida, A. R., Zaragoza, B. & Freitas, A. A. Indexation as a novel mechanism of lymphocyte homeostasis: the number of CD4+CD25+ regulatory T cells is indexed to the number of IL-2-producing cells. *J Immunol* **177**, 192-200 (2006).
68. Amado, I. F. *et al*. Quorum sensing in CD4+ T cell homeostasis: IL-2 coordinates IL-2 producing and regulatory T cell interplay. *J Exp Med* **210**, 2707-2720 (2013).
69. Guy, C. S. *et al*. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. *Nat Immunol* **14**, 262-270 (2013).
70. Zeng, H. *et al*. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* **499**, 485-490 (2013).
71. Chen, Q. *et al*. IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. *J Immunol* **186**, 6329-6337 (2011).
72. Suffner, J. *et al*. Dendritic cells support homeostatic expansion of Foxp3+ regulatory T cells in Foxp3.LuciDTR mice. *J Immunol* **184**, 1810-1820 (2010).
73. Cretney, E. *et al*. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* **12**, 304-311 (2011).
74. Delgoffe, G. M. *et al*. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature* **501**, 252-256 (2013).
75. Carrette, F. & Surh, C. D. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin Immunol* **24**, 209-217 (2012).
76. Dummer, W. *et al*. Autologous regulation of naive T cell homeostasis within the T cell compartment. *J Immunol* **166**, 2460-2468 (2001).
77. Walker, L. S. Treg and CTLA-4: Two intertwining pathways to immune tolerance. *J Autoimmun* **45**, 49-57 (2013).
78. Benson, A. *et al*. Microbial infection-induced expansion of effector T cells overcomes the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism. *J Immunol* **188**, 800-810 (2012).
79. Oldenhove, G. *et al*. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* **31**, 772-786 (2009).
80. Gratz, I. K. *et al*. Cutting Edge: memory regulatory T cells require IL-7 and not IL-2 for their maintenance in peripheral tissues. *J Immunol* **190**, 4483-4487 (2013).
81. Raynor, J. *et al*. IL-15 Fosters Age-Driven Regulatory T Cell Accrual in the Face of Declining IL-2 Levels. *Frontiers in immunology* **4**, 161 (2013).
82. Sadlack, B. *et al*. Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. *Eur J Immunol* **25**, 3053-3059 (1995).

83. Schorle, H. *et al*. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* **352**, 621-624 (1991).
84. Wolf, M., Schimpl, A. & Hunig, T. Control of T cell hyperactivation in IL-2-deficient mice by CD4(+)CD25(-) and CD4(+)CD25(+) T cells: evidence for two distinct regulatory mechanisms. *Eur J Immunol* **31**, 1637-1645 (2001).
85. Willerford, D. M. *et al*. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* **3**, 521-530 (1995).
86. Malek, T. R. *et al*. Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. *J Immunol* **164**, 2905-2914 (2000).
87. Suzuki, H. *et al*. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. *Science* **268**, 1472-1476 (1995).
88. Burchill, M. A. *et al*. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* **178**, 280-290 (2007).
89. Snow, J. W. *et al*. Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice. *J Immunol* **171**, 5042-5050 (2003).
90. Sharfe, N., Dadi, H. K., Shahar, M. & Roifman, C. M. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. *Proc Natl Acad Sci U S A* **94**, 3168-3171 (1997).
91. Roifman, C. M. Human IL-2 receptor alpha chain deficiency. *Pediatric research* **48**, 6-11 (2000).
92. Cohen, A. C. *et al*. Cutting edge: Decreased accumulation and regulatory function of CD4+ CD25(high) T cells in human STAT5b deficiency. *J Immunol* **177**, 2770-2774 (2006).
93. Yamanouchi, J. *et al*. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nature genetics* **39**, 329-337 (2007).
94. Lowe, C. E. *et al*. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. *Nature genetics* **39**, 1074-1082 (2007).
95. International Consortium for Systemic Lupus Erythematosus, *et al*. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nature genetics* **40**, 204-210 (2008).
96. Blazar, B. R., Murphy, W. J. & Abedi, M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol* **12**, 443-458 (2012).
97. Gaidot, A. *et al*. Immune reconstitution is preserved in hematopoietic stem cell transplantation coadministered with regulatory T cells for GVHD prevention. *Blood* **117**, 2975-2983 (2011).
98. Wysocki, C. A. *et al*. Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. *Blood* **106**, 3300-3307 (2005).
99. Safinia, N. *et al*. Promoting transplantation tolerance; adoptive regulatory T cell therapy. *Clinical and experimental immunology* **172**, 158-168 (2013).

100. Edinger, M. *et al*. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* **9**, 1144-1150 (2003).
101. Shin, H. J. *et al*. Rapamycin and IL-2 reduce lethal acute graft-versus-host disease associated with increased expansion of donor type CD4+CD25+Foxp3+ regulatory T cells. *Blood* **118**, 2342-2350 (2011).
102. Prinz, I. & Koenecke, C. Therapeutic potential of induced and natural FoxP3(+) regulatory T cells for the treatment of Graft-versus-host disease. *Archivum immunologiae et therapiae experimentalis* **60**, 183-190 (2012).
103. Mielke, S. *et al*. Reconstitution of FOXP3+ regulatory T cells (Tregs) after CD25-depleted allotransplantation in elderly patients and association with acute graft-versus-host disease. *Blood* **110**, 1689-1697 (2007).
104. Magenau, J. M. *et al*. Frequency of CD4(+)CD25(hi)FOXP3(+) regulatory T cells has diagnostic and prognostic value as a biomarker for acute graft-versus-host-disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **16**, 907-914 (2010).
105. Ukena, S. N. *et al*. Human regulatory T cells in allogeneic stem cell transplantation. *Blood* **118**, e82-92 (2011).
106. Ukena, S. N. *et al*. Acute but not chronic graft-versus-host disease is associated with a reduction of circulating CD4(+)CD25 (high)CD127 (low/-) regulatory T cells. *Annals of hematology* **90**, 213-218 (2011).
107. Brunstein, C. G. *et al*. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* **117**, 1061-1070 (2011).
108. Di Ianni, M. *et al*. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117**, 3921-3928 (2011).
109. Koreth, J. *et al*. Interleukin-2 and regulatory T cells in graft-versus-host disease. *The New England journal of medicine* **365**, 2055-2066 (2011).
110. Matsuoka, K. *et al*. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Science translational medicine* **5**, 179ra143 (2013).
111. Barthlott, T. *et al*. CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *International immunology* **17**, 279-288 (2005).
112. Sitrin, J. *et al*. Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *J Exp Med* **210**, 1153-1165 (2013).
113. Franceschini, D. *et al*. PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *J Clin Invest* **119**, 551-564 (2009).
114. Dupont, C. D., Christian, D. A. & Hunter, C. A. Immune response and immunopathology during toxoplasmosis. *Seminars in immunopathology* **34**, 793-813 (2012).
115. Wojno, E. D. *et al*. A role for IL-27 in limiting T regulatory cell populations. *J Immunol* **187**, 266-273 (2011).
116. Quintana, F. J. *et al*. Aiolos promotes TH17 differentiation by directly silencing Il2 expression. *Nat Immunol* **13**, 770-777 (2012).

117. Venet, F. *et al*. Regulatory T cell populations in sepsis and trauma. *Journal of leukocyte biology* **83**, 523-535 (2008).
118. Cavassani, K. A. *et al*. The post sepsis-induced expansion and enhanced function of regulatory T cells create an environment to potentiate tumor growth. *Blood* **115**, 4403-4411 (2010).
119. Venet, F. *et al*. Increased circulating regulatory T cells (CD4(+)CD25 (+)CD127 (-)) contribute to lymphocyte anergy in septic shock patients. *Intensive care medicine* **35**, 678-686 (2009).
120. Ono, S. *et al*. Removal of increased circulating CD4+CD25+Foxp3+ regulatory T cells in patients with septic shock using hemoperfusion with polymyxin B-immobilized fibers. *Surgery* **153**, 262-271 (2013).