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Mechanisms of impaired regulation by $CD4^+CD25^+FOXP3^+$ regulatory T cells in human autoimmune diseases

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Abstract

A lack of regulatory T (T_{Reg}) cells that express CD4, CD25 and forkhead box P3 (FOXP3) results in severe autoimmunity in both mice and humans. Since the discovery of T_{Reg} cells, there has been intense investigation aimed at determining how they protect an organism from autoimmunity and whether defects in their number or function contribute to the development of autoimmunity in model systems. The next phase of investigation — that is, to define the role that defects in T_{Reg} cells have in human autoimmunity — is now underway. This Review summarizes our progress so far towards understanding the role of $CD4^+CD25^+FOXP3^+$ T_{Reg} cells in human autoimmune diseases and the impact that this knowledge might have on the diagnosis and treatment of these diseases.

Regulatory T (T_{Reg}) cells, defined by the expression of CD4, CD25 and the transcription factor forkhead box P3 (FOXP3), have a central role in protecting an individual from autoimmunity. This role was first identified in mice in which the absence of T_{Reg} cells, or the depletion of T_{Reg} cells, resulted in the development of autoimmune gastritis, thyroiditis, diabetes and inflammatory bowel disease (IBD)[1,2]. Subsequently, numerous studies in animal models of autoimmunity showed that defects in $CD4^+CD25^+FOXP3^+$ T_{Reg} cells can contribute to the development of autoimmunity and that the disease could be reversed by the adoptive transfer of T_{Reg} cells (reviewed in REF. [3]). This was followed by studies identifying the presence of T_{Reg} cells in human peripheral blood and their ability to suppress T cell proliferation *in vitro*[4–6]. The importance of T cell regulation in human disease is highlighted by the severe inflammation and autoimmunity that occurs in individuals who suffer from immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). These individuals develop a broad range of autoantibodies, insulin-dependent diabetes, thyroiditis, eczema, haemolytic anaemia and IBD, and in the absence of a bone marrow transplant, these patients die at an early age (reviewed in REF. [1]). These observations have driven a search for mechanisms of defective T cell regulation in human autoimmunity. In this Review, I discuss the evidence supporting the involvement of impaired T cell regulation in auto-immunity and our current understanding of the source of these regulatory defects. This is considered in the context of several autoimmune and inflammatory diseases: type 1 diabetes, multiple sclerosis, systemic lupus erythematosus

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Competing interests statement
The author declares no competing financial interests.

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(SLE), rheumatoid arthritis, IBD and psoriasis. Although multiple types of regulatory T cell have been described[7], I focus on the $CD4^{+}CD25^{+}FOXP3^{+}$ T_{Reg} cell subset in this Review.

Mechanisms of impaired T cell regulation

To address the question of whether immune suppression by T_{Reg} cells is impaired in the setting of human auto-immune disease, it is important to recognize the potential means by which such a defect may occur. As shown in FIG. 1 and described in BOX 1, defects in the number and function of T_{Reg} cells, as well as a resistance of effector T cells to T_{Reg} cell-mediated suppression, could each contribute to failed T cell regulation. Each of these defects has been shown to contribute to the development of autoimmunity in various model systems. In these models, the underlying mechanisms by which these defects in regulation occur have also been investigated. Such studies indicate that cell-intrinsic defects in effector T cells, $CD4^{+}FOXP3^{+}$ T cells and antigen-presenting cells (APCs), as well as alterations in the composition of the inflammatory milieu, can contribute to failed tolerance to self. Here I address how each of these potential breaches in regulation can be assessed in humans, the current evidence that defects in these pathways are present and the challenges that must be overcome to further define the mechanisms of impaired tolerance in human autoimmune disease.

Box 1

Factors that affect T cell regulation in autoimmunity

The number of regulatory T (T_{Reg}) cells found in individuals with autoimmune disease is influenced by T_{Reg} cell development, persistence and proliferation in the periphery and homing to the site of inflammation. Genetic factors are likely to have the strongest impact on thymic output of T_{Reg} cells. Maintenance of T_{Reg} cells in the periphery is a dynamic process, influenced in part by conditions that favour the induction of T_{Reg} cells in the periphery and support their proliferation and survival. Factors that favour the thymic development, peripheral growth and survival of T_{Reg} cells have been shown to have an effect on forkhead box P3 (FOXP3) expression[11,12,110]. Such factors include CD28, transforming growth factor-β (TGFβ), dendritic cells and the common cytokine receptor γ-chain (γ_c) cytokines (interleukin-2 (IL-2), IL-4, IL-7 and IL-15), which signal through signal transducer and activator of transcription 5 (STAT5).

T_{Reg} cell dysfunction in autoimmune disease may be due to a defect in one of the many mechanisms through which T_{Reg} cells function (reviewed in REF. [22]). This could occur through inadequate expression of cell surface molecules that are known to be involved in contact-dependent suppression (such as cytotoxic T lymphocyte antigen 4 (CTLA4), CD39, lymphocyte activation gene 3 (LAG3), granzyme A and CD95 (also known as FAS)) or as a result of a failure to produce the soluble factors (such as TGFβ, IL-10 and IL-35) that are involved in some aspects of suppression. Underlying genetic factors may influence these mechanisms. In addition, the composition of the local milieu, including the types of antigen-presenting cells and cytokines (such as tumour necrosis factor (TNF)[29,111], IL-4 (REF. [112]), IL-6 (REF. [29]), IL-12 (REF. [113]), IL-7, IL-15 (REF. [114]) and IL-21 (REF. [28])), can influence T_{Reg} cell function.

Multiple mechanisms by which effector T cells resist T_{Reg} cells have been proposed (reviewed in REF. [31]). Cell-intrinsic resistance to suppression has been shown to occur in $CD4^{+}$ memory T cells and T helper 17 (T_H17) cells[115]. The cytokines IL-2, IL-4, IL-7 and IL-15 support the proliferation of effector T cells in the presence of T_{Reg} cells, indicating that despite the favourable roles of these cytokines in T_{Reg} cell homeostasis[110], the presence of these cytokines in the short term allows effector T cells to bypass suppression by T_{Reg} cells. In addition, several members of the TNF receptor

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family have been implicated in this process: antibody specific for OX40 (also known as TNFRSF4) abrogates suppression when bound to effector T cells[116], and ligation of 4-1BB (also known as TNFRSF9) results in suppression-resistant effector T cells[117].

Inadequate numbers of T_{Reg} cells

In mouse models, the concept that inadequate numbers of T_{Reg} cells may contribute to autoimmunity is supported by the occurrence of aggressive autoimmunity in scurfy mice and is indirectly implied by the successful treatment of autoimmunity in mice through the adoptive transfer of wild-type T_{Reg} cells[8,9]. In addition, there is evidence from mouse models that, under the appropriate conditions, T_{Reg} cells can be induced in the periphery, and these T_{Reg} cells may protect from the development of autoimmunity[8–10]. Multiple factors influence the homeostasis and induction of T_{Reg} cells in the periphery, including CD28, interleukin-2 (IL-2), transforming growth factor-β (TGFβ)[11] and dendritic cells (DCs)[12].

Evidence that an inadequate number of T_{Reg} cells leads to autoimmunity in humans is most clearly shown in patients with IPEX, who completely lack T_{Reg} cells as a result of a mutation in *FOXP3* (REF. [13]). However, most patients with autoimmune disease probably have a more modest reduction in T_{Reg} cells. In these common diseases, the challenge is to determine whether the number of T_{Reg} cells is inadequate at the site of inflammation and whether this is due to systemic factors or factors in the local tissue milieu. In human disease, the task of enumerating T_{Reg} cells has been complicated by two main issues. The first issue is deciding which cells to count. This is complicated by the lack of a cell marker that is unique to T_{Reg} cells and the multiplicity of T_{Reg} cell subsets (BOX 2). The second issue is the extent to which the peripheral blood reflects the global number of T_{Reg} cells in the body and, more specifically, their number in inflamed tissues.

Box 2

Regulatory T cell subsets: origins, phenotypes and functions

Multiple T cell subsets with suppressive functions have been identified. The $CD4^+$ T cell subsets are defined by origin, function, and the expression of cell surface markers and the transcription factor forkhead box P3 (FOXP3).

Type 1 regulatory T (T_R1) cells are induced in the periphery, suppress T cell proliferation through the production of interleukin-10 (IL-10) and transforming growth factor-β (TGFβ)[118] and do not have a unique cell marker but are identified by their production of IL-10 and not pro-inflammatory cytokines.

T helper 3 (T_H3) cells are a regulatory T cell population that originates in the periphery and mediates suppression through the secretion of TGFβ; similar to T_R1 cells, they do not have a unique cell surface marker[119].

$CD4^+CD25^+FOXP3^+$ regulatory T (T_{Reg}) cells can be divided into two groups: thymus-derived natural T_{Reg} cells and periphery-induced adaptive T_{Reg} cells. Both populations express FOXP3 and suppress immune responses through contact-dependent mechanisms and the production of soluble factors, including the cytokines TGFβ, IL-10 and IL-35 (REFS [18,22]). Thymus-derived $CD4^+CD25^+FOXP3^+$ T_{Reg} cells are stable with respect to retaining regulatory function and FOXP3 expression in the periphery. They are unique in that their *FOXP3* locus is demethylated[120] and they express the transcription factor Helios[20]. Adaptive T_{Reg} cells can be induced in the periphery from a $CD4^+FOXP3^-$ T cell population following T cell receptor stimulation in the presence of TGFβ. These cells express the same cell surface markers as natural T_{Reg} cells and suppress immune

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responses through cytokines and contact-dependent mechanisms. They can be distinguished from natural T_{Reg} cells based on *FOXP3* DNA methylation patterns and their lack of Helios expression.

It has now become clear that the $FOXP3^+$ T cell population is composed of several populations that are defined by the expression of CD25, CD45RA and FOXP3. Miyara *et al.*[21] defined these populations as a naive T_{Reg} cell population that is $CD25^{hi}CD45RA^+FOXP3^{hi}$, an effector T_{Reg} cell population that is $CD25^{hi}CD45RA^-FOXP3^{hi}$ and a non-regulatory $FOXP3^+$ population that is $CD25^{hi}CD45RA^-FOXP3^{low}$. Our growing understanding of the complexity of T_{Reg} cells indicates that we must continue to consider how alterations in the composition, function and stability of the T_{Reg} cell pool may contribute to autoimmunity. When examining the literature, understanding how T_{Reg} cells are identified and isolated has important implications for interpretation of the data. New markers will improve these studies in the future.

T_{Reg} cells were first defined on the basis of their expression of CD25, which forms part of the high-affinity IL-2 receptor. Among the $CD4^+CD25^+$ T cell population is a subset of cells that express CD25 at a high level — approximately 4% of the $CD4^+$ population in human blood — and most of this population has regulatory function. Unfortunately, the definition of T_{Reg} cells based on the level of CD25 expression has not been consistently reported in the literature, and this makes comparisons between studies difficult. Furthermore, CD25 is also expressed by recently activated T cells, resulting in the inclusion of $CD4^+CD25^+$ effector T cells in the T_{Reg} cell population. With the discovery that expression of FOXP3 has a central role in the differentiation and maintenance of T_{Reg} cells[14,15], the use of flow cytometry-based analysis of FOXP3 expression in T cells became the gold standard for defining T_{Reg} cells. However, it then became evident that FOXP3 can also be expressed by effector T cells following activation[16], raising the possibility that any assessment of T_{Reg} cell number or function may include recently activated effector T cells in the T_{Reg} cell population. Furthermore, as FOXP3 is a nuclear protein, assessment of its expression in T cells requires fixation and permeabilization of the cells, resulting in an inability to obtain viable cells for further functional analysis. In the past few years, additional markers, such as CD127 (also known as IL-7Rα)[17], have been identified that assist in the distinction of effector T cells from T_{Reg} cells and facilitate the experimental purification of T_{Reg} cells[18].

More recently, the ability to distinguish thymus-derived natural T_{Reg} cells from those that are induced in the periphery (BOX 2) has been facilitated by the discovery that the presence of specific demethylated sites in the *FOXP3* promoter[19] and the expression of the nuclear protein Helios are unique to thymus-derived natural T_{Reg} cells[20]. The use of these markers, as well as the use of intracellular cytokine staining, has led to the discovery of discrete T_{Reg} cell subsets that have unique functional characteristics[21,22]. Defining these T_{Reg} cell subsets may be of central importance when enumerating T_{Reg} cells in autoimmunity.

Defining defects in T_{Reg} cell function

Identifying defects in the function of T_{Reg} cells is made difficult both by the multiple mechanisms used by T_{Reg} cells to suppress inflammation (reviewed in REFS [23,24]) and by the manner in which suppression is measured. In addition, assessment of T_{Reg} cell function in humans requires the use of *in vitro* assays that, owing to the rarity of T_{Reg} cells in the peripheral blood, must be carried out with low cell numbers, limiting the type and quality of assays that can be done. Currently, assays of T_{Reg} cell function address the ability of T_{Reg} cells to inhibit the proliferation of, or cytokine production by, co-cultured effector T cells (BOX 3). Most co-culture assays are carried out with autologous responder T cells and

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APCs; such studies can define defects in suppression but do not specifi cally test the function of T_{Reg} cells. To determine whether the source of impaired suppression is intrinsic to the T_{Reg} cells, investigators have used assays that examine the suppressive function of an individual's T_{Reg} cells using effector T cell and/or APC populations that are isolated from healthy controls. Although these assays provide insight into potential T_{Reg} cell defects, they cannot account for the impact of the local milieu on T_{Reg} cell function.

Box 3

In vitro assays of suppression

Studies of regulatory T (T_{Reg}) cell function in human autoimmune diseases have examined the proliferation of responder cells in response to polyclonal activation in co-cultures with T_{Reg} cells isolated from populations of peripheral blood mononuclear cells. Measurements are based on the incorporation of ^{3}H or on the dilution of the fluorescent label 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Evaluation of cytokine production by the responder cells can also be carried out in conjunction with these assays. CFSE dilution is in several ways a superior approach for examining T_{Reg} cell-mediated suppression, as it reflects the number of cell divisions throughout the culture period, whereas ^{3}H incorporation assays only reflect the level of proliferation that occurs during the period in which the ^{3}H is present in the culture (typically 12–16 hours). The use of CFSE also has the advantage of allowing simultaneous analysis of cell surface markers and cytokines to better define the character of the proliferating responder cells. However, the number of cells that is required for CFSE assays is much greater than that needed for ^{3}H incorporation assays, making CFSE assays less useful when only small amounts of blood are available. The outcome of these studies is influenced by the type and strength of the stimulus used, and interpretation of any of these studies should take this into account[41]. In addition, the manner by which the T_{Reg} cells are isolated can influence the composition of the T_{Reg} cell pool and therefore the degree of inhibition. The type of selection method (bead based or flow cytometry based) and the stringency of selection (based on the level of CD25 expression and the use of additional T_{Reg} cell markers) can lead to large differences in the percentage of isolated cells that express forkhead box P3 (FOXP3) and in the level of suppression. Alternative approaches that measure antigen-specific or pathogenic T cell responses in the presence or absence of T_{Reg} cells have also been used. The limitation of these assays is typically the weak response of $CD4^+$ T cells to a self antigen owing to low precursor frequency, which is further complicated by the inability to control for the T_{Reg} cell/effector T cell ratio in the culture.

Measuring resistance of effector T cells to suppression

The resistance of effector T cells to T_{Reg} cells has been observed in several animal models of autoimmunity. In these models, inflammation and tissue destruction progress despite the presence of functional T_{Reg} cells at the site of inflammation. Such findings suggest that a resistance of effector T cells to T_{Reg} cells may contribute to disease progression. This phenomenon has been described in two mouse models of diabetes (non-obese diabetic mice (NOD mice)[25–27] and DO11.10 RIP-mOVA mice[28]), in experimental autoimmune encephalomyelitis (EAE)[29] and in the MRL–lpr mouse model of SLE[30].

There seem to be multiple ways in which effector T cells can become resistant to suppression. The mechanisms by which this occurs include T cell-intrinsic defects, alterations in the strength of T cell activation and exposure to T cell growth factors (reviewed in REF. [31]). To determine whether this phenomenon occurs in humans, an assay

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system that allows for the assessment of effector T cell suppression by allogeneic T_{Reg} cells was required. Such assays have been established and have provided evidence of effector T cell resistance to suppression in several human autoimmune diseases (see below).

Each of the defects in regulation described above may contribute, either in isolation or in combination, to the development of human autoimmune disease. The task of determining the extent and character of these defects in human disease is challenging. Despite these limitations, when studies of human subjects are carried out with a consistent approach to analysis and a well-matched control population, it is possible to determine the potential defects in T cell regulation in an individual. This knowledge then allows the development of hypotheses and the determination of disease mechanisms that are relevant to human disease.

T_{Reg} cells and type 1 diabetes

Type 1 diabetes is an immune-mediated disease that results in inflammation and destruction of the pancreatic islet cells, resulting in a lifelong need for insulin. A role for T_{Reg} cells in autoimmune diabetes is evident in individuals with IPEX, in which the absence of T_{Reg} cells results in enhanced susceptibility to diabetes. In addition, in NOD mice (a spontaneous model of type 1 diabetes), T_{Reg} cell numbers and FOXP3 expression decrease with the age of the animals despite ongoing autoimmunity[32], and over time the pathogenic effector T cells in the pancreatic islets become resistant to suppression[26–28]. Adoptive transfer of T_{Reg} cells ameliorates disease in NOD mice[9], as does treatment with the growth factor IL-2, which increases FOXP3 expression and T_{Reg} cell number[32].

Whether the number of T_{Reg} cells is reduced or normal in patients with type 1 diabetes remains an issue of controversy. The initial analyses of T_{Reg} cell numbers (using CD25 expression to define T_{Reg} cells) reported a significant decrease in $CD4^{+}CD25^{+}$ T_{Reg} cell numbers in individuals with newly diagnosed or established disease[33]. This study was followed by a series of other studies that found no difference in the number of $CD4^{+}CD25^{+}$ or $CD4^{+}CD25^{hi}$ T cells in the peripheral blood of patients with type 1 diabetes compared with controls[34–37]. Later studies using nuclear staining of FOXP3 as a marker of T_{Reg} cells also did not observe differences between healthy controls and patients[35]. Moreover, a recent study[38] found no significant difference in the level of demethylation at the T_{Reg} cell-specific demethylated region (TSDR) of *FOXP3* in T_{Reg} cells from patients with type 1 diabetes compared with controls and, furthermore, the authors did not find differences in the distribution of T_{Reg} cells between the $CD4^{+}FOXP3^{+}CD45RA^{+}$ and $CD4^{+}FOXP3^{+}CD45RO^{+}$ T_{Reg} cell subsets. Another recent study examining these T_{Reg} cell subsets in individuals with new-onset diabetes reports a relative increase in the $CD45RA^{-}FOXP3^{low}$ T cell population compared with controls and shows that these cells produce IL-17 and are non-regulatory. Thus, in early disease there may be an inadequate number of functional T_{Reg} cells[39].

Although the total number of circulating T_{Reg} cells seems to be normal in patients with type 1 diabetes, it is possible that the persistence and function of T_{Reg} cells are impaired at sites of inflammation. A recent study showed that T_{Reg} cells from patients with type 1 diabetes had a reduced capacity to respond to IL-2, and the authors correlated this defect in IL-2-induced signalling with a loss of FOXP3 expression[38]. This finding mirrors the loss of FOXP3 expression by T_{Reg} cells infiltrating the islets in NOD mice owing to a decrease in the levels of available IL-2 (REF. [32]). A histological study of islets that were isolated from patients with new-onset type 1 diabetes immediately post-mortem found that $FOXP3^{+}$ T cells were only rarely present in the islets; this is consistent with the idea that there are inadequate numbers of T_{Reg} cells in the islets of individuals with type 1 diabetes[40]. Currently, additional studies that are designed to enumerate T_{Reg} cells at the sites of

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inflammation — the pancreatic islets and draining lymph nodes — are underway and may shed more light on this issue.

A functional defect in T_{Reg} cells isolated from individuals with type 1 diabetes has been shown by several investigators (reviewed in REF. [41]). These studies assessed the impact of $CD4^+CD25^+$ T_{Reg} cells, isolated by beads or flow cytometry, on autologous responder $CD4^+$ T cells following nonspecific activation. Although the results have been mixed, most of these studies showed a decrease in the degree of T_{Reg} cell-mediated inhibition[34,36,37]. All of the suppression assays were carried out using autologous effector T cells, so they do not specifically define the defect as being intrinsic to the T_{Reg} cells. More recently, defects that are intrinsic to a T_{Reg} cell population in type 1 diabetes have been identified. In these studies[42,43], the T_{Reg} cells of several individuals with type 1 diabetes were unable to suppress effector T cells from a healthy control.

The role of effector T cell resistance in type 1 diabetes has also been assessed by two independent groups. Studies by my group showed effector T cell resistance to suppression in type 1 diabetes, using a 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assay. We found that the resistance was intrinsic to the effector T cells and occurred irrespective of the source of T_{Reg} cells (natural T_{Reg} cells or inducible T_{Reg} cells)[42]. Similar resistance to suppression was also shown by Lawson *et al.*[43] using a ^3H-based assay system. Both of these investigations studied individuals with long-standing type 1 diabetes, raising the important issue of whether the defect is a cause or an effect of disease.

In summary, the current evidence does not indicate a global deficiency in T_{Reg} cell numbers in type 1 diabetes but does not rule out a more focal deficiency in the T_{Reg} cell compartment, with respect to either a subset of T_{Reg} cells or the specificity of T_{Reg} cells for islet antigens. Multiple studies show that suppression is impaired and indicate that this is, in part, due to the resistance of effector T cells to suppression by T_{Reg} cells.

T_{Reg} cells and multiple sclerosis

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Multiple sclerosis is an autoimmune disease characterized by inflammatory lesions and degeneration in the central nervous system (CNS). The pathogenic $CD4^+$ T cells in multiple sclerosis target myelin-based antigens, have the ability to migrate to the CNS and have unique effector functions[44]. Although the inflammatory components involved in multiple sclerosis are numerous, defects in T_{Reg} cells as a component of this disease are implicated by both the mouse model of multiple sclerosis (EAE) and the human disease.

Animals that express a transgenic T cell receptor specific for myelin basic protein (MBP) and lack recombination-activating genes (RAGs) spontaneously develop EAE owing to an absence of T_{Reg} cells[14], indicating a role for T_{Reg} cells in the prevention of EAE. Further studies have shown an increase in susceptibility to EAE following depletion of T_{Reg} cells using CD25-specific antibody, and adoptive transfer of T_{Reg} cells reduces the incidence of EAE[14,45]. However, after the disease is established, the ability of T_{Reg} cells to control inflammation in the CNS is controversial, despite their ability to migrate to the CNS. One explanation for this is the resistance of effector T cells in the CNS to T_{Reg} cell-mediated suppression during active disease owing to the production of IL-6 and tumour necrosis factor (TNF)[45].

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As for other human autoimmune diseases, the number of T_{Reg} cells in the peripheral blood is the most extensively investigated aspect of T cell regulation in multiple sclerosis. Studies have assessed T_{Reg} cell number in patients with active or inactive disease and those receiving therapy. In most of these studies, no differences were observed in the percentage of T_{Reg} cells (defined as $CD4^+CD25^{hi}$ cells) in the blood of healthy control individuals or

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subjects with multiple sclerosis, irrespective of disease activity[46–52]. However, the number of T_{Reg} cells has been found to be increased in the cerebrospinal fluid compared with the number in the peripheral blood in patients with multiple sclerosis[49,53]. In two studies[54,55], the number of T_{Reg} cells (defined as $CD4^+CD25^+FOXP3^+$ cells) was found to be increased in patients with multiple sclerosis; in the study by de Andrés *et al.*[55], this increase normalized after 6 months of therapy with interferon-β (IFNβ). Only one study found a decrease in $CD4^+CD25^+FOXP3^+$ T cell numbers compared with healthy controls[53]; in this case, the decrease was unique to subjects with relapsing–remitting multiple sclerosis and normal cell numbers were restored following treatment with IFNβ. An important aspect of this study was that the authors observed that the percentage of $FOXP3^+$ cells was stable over a 1-year period in a subset of untreated patients with relapsing–remitting multiple sclerosis[53].

More recent studies have identified alterations in the composition of the T_{Reg} cell pool in multiple sclerosis. Papers by Haas *et al.*[48] and Venken *et al.*[51] both reported a decrease in the naive or recent thymic immigrant T_{Reg} cells, a population of T_{Reg} cells that has enhanced suppressive function. A decrease in the percentage of $CD4^+CD25^{hi}$ cells that express CD39, a molecule that has been linked to T_{Reg} cell function in mice, has also been described[52]. In addition, subjects with multiple sclerosis have been reported to have an increase in the $CD127^+$ population of $FOXP3^+$ T_{Reg} cells, a subset that is not suppressive[50]. One interesting finding that also questions the importance of T_{Reg} cell numbers in multiple sclerosis was made in patients with multiple sclerosis who received daclizumab (Zenapax; Hoffmann-La Roche), a monoclonal antibody that targets CD25 and results in a reduction in $FOXP3^+$ cell numbers. The authors of this study did not observe a correlation between the clinical response to the treatment and the decrease in $FOXP3^+$ cells in these patients[56]. Overall, there is no evidence for inadequate numbers of T_{Reg} cells in established multiple sclerosis, but more recent studies suggest that alterations in the composition of the T_{Reg} cell population may have a role in the disease.

There has also been extensive study of T_{Reg} cell function in multiple sclerosis. Such studies consistently show impaired suppression when T_{Reg} cells are incubated with autologous responder T cells, using co-cultures in which proliferation is measured by either ^3H incorporation or CFSE dilution[46,50,57–59]. In two of these studies[46,57], the suppression of secretion of IFNγ by effector T cells has also been shown to be diminished in patients with multiple sclerosis. In addition, two studies have examined the suppression of myelin-specific T cells and found a decrease in the suppression of myelin oligodendrocyte glycoprotein (MOG)- and MBP-specific responses in patients with multiple sclerosis compared with controls[54,57]. However, it is worth noting that no correlation between T_{Reg} cell function and disease activity has been identified.

Several sources of regulatory defects have been observed in multiple sclerosis. Contamination of the studied T_{Reg} cell population with non-suppressive cells has been described by two groups[50,58]. Michel *et al.*[50] found that the level of suppression did not differ between multiple sclerosis subjects and control subjects if only the top 2% of $CD4^+CD25^+$ cells was used in suppression assays, thereby excluding a subset of $CD127^+$ cells that produce pro-inflammatory cytokines. A defect in the production of the regulatory cytokine IL-10 by T_{Reg} cells has also been implicated by Astier *et al.*[59], who found that following activation of $CD4^+$ T cells from patients with multiple sclerosis using CD3-specific antibody and CD46-specific antibody, IL-10 production is reduced. However, this was not seen when the cells were activated with CD3-specific antibody and CD28-specific antibody and thus it does not explain the impaired suppression seen in other studies but suggests yet another mechanism by which suppression might be impaired in multiple sclerosis[59].

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Three studies have addressed whether effector T cells are resistant to suppression in multiple sclerosis[46,48,57]. None of these studies observed defective suppression of effector T cells from patients with multiple sclerosis by T_{Reg} cells from controls. However, these experiments were carried out with a very limited number of samples (one to three individuals were studied in each investigation). Given that effector T cell resistance has been observed in EAE[45], that multiple sclerosis is a heterogeneous disease and that recent studies have described effector T cell resistance in other autoimmune diseases[42,60,61], this area would benefit from further investigation.

Overall, T_{Reg} cells do not seem to be reduced in number in multiple sclerosis, but clear defects in suppression have been identified. These defects may, in part, be due to the composition and function of the T_{Reg} cell pool.

T_{Reg} cells and SLE

SLE is a systemic autoimmune disease that is characterized by the presence of autoantibodies and immune complexes that target multiple organ systems, including the skin, joints, kidneys and CNS. Unlike the autoimmune diseases discussed earlier, there are many target tissues in SLE, not just one, and it is thought to be largely a B cell-mediated disease. Nevertheless, deficiency of T_{Reg} cells results in the development of lupus-like characteristics, including glomerulonephritis and the development of DNA-specific antibodies. These findings indicate that failure of T_{Reg} cell-mediated suppression may have a role in human SLE[8].

Many studies have assayed the number of T_{Reg} cells in the peripheral blood of individuals with SLE (reviewed in REF. [62]). Most, but not all, of these studies have shown that the percentage of $CD4^+CD25^{hi}$ cells is decreased in patients with SLE. This decrease in $CD4^+CD25^{hi}$ T_{Reg} cells was found to be inversely correlated with disease activity in several studies[63–67], no correlation was reported in one study[68] and, in a few cases, an increase in T_{Reg} cells has been observed[60]. However, no increase was observed when FOXP3 was used as a marker of T_{Reg} cells[60], and an increase in T_{Reg} cell numbers following treatment with corticosteroids has been observed by two groups[69,70].

The function of T_{Reg} cells in SLE has been assessed by multiple groups. Several of these groups have found no defect in function when very stringent methods of isolation and selection of T_{Reg} cells were used[21,66]. However, most studies of function, irrespective of the method of T_{Reg} cell selection, have shown a defect in their suppressive activity, mainly based on measures of effector T cell proliferation, although measures of IFNγ production by effector T cells have also confirmed these findings[60,71,72]. In several studies, the defect in suppression correlates with disease activity[60,71]. The source of defective suppression has been attributed to both the APCs and the T_{Reg} cells. Yan *et al.*[73] showed that suppression was defective only in the presence of APCs and was linked to their production of IFNα. T_{Reg} cell-intrinsic defects have also been linked to increased sensitivity of these cells to cell death mediated by the death receptor CD95 (also known as FAS)[21] and to a diminished expression of FOXP3 due to a relative lack of IL-2 production in SLE[72].

The MRL–lpr mouse model of SLE has been shown to be associated with effector T cells that are resistant to suppression by T_{Reg} cells[30]. Several early T_{Reg} cell studies that addressed the issue of effector T cell resistance in SLE could not detect this defect[72–74], but two recent studies have shown that effector T cells can evade suppression in SLE. Venigalla *et al.*[60] observed a resistance to suppression in a cohort of patients with active SLE, whereas Vargas-Rojas *et al.*[61] found effector T cell resistance in SLE irrespective of disease activity. The replication of these findings by two groups indicates that effector T cell resistance is a probable component of the loss of tolerance in SLE.

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In SLE, defects in the number and function of T_{Reg} cells and in the resistance of effector T cells to suppression have been established. The observations of numerous possible regulatory defects may reflect the systemic character of this disease or a more significant role for T_{Reg} cells in SLE compared with their role in other autoimmune diseases.

T_{Reg} cells and rheumatoid arthritis

As in other autoimmune diseases, the experimental model of rheumatoid arthritis (collagen-induced arthritis) is exacerbated by depletion of T_{Reg} cells[75]. However, unlike the diseases discussed earlier, the target tissue in rheumatoid arthritis — the synovium — can be obtained from patients with disease. This has allowed investigators to analyse the number and function of T_{Reg} cells not only in the peripheral blood of these patients but also in the diseased tissue. Several analyses of T_{Reg} cell numbers in the peripheral blood of subjects with rheumatoid arthritis have produced differing results. In established disease, the $CD4^{+}CD25^{hi}$ population has been shown to be no different from that of controls[76,77], whereas a modest decrease in T_{Reg} cells was reported for untreated patients with early stage rheumatoid arthritis[78]. These findings contrast with observations by Han *et al.*[79], who reported an increase in the relative and absolute numbers of T_{Reg} cells (based on $CD4^{+}CD25^{hi}FOXP3^{+}$ staining) in the peripheral blood of patients with rheumatoid arthritis compared with numbers in controls. Despite these differences, there is general agreement that the percentage of T_{Reg} cells is higher in the synovial fluid in patients with rheumatoid arthritis than in controls[76–78].

Initial studies of the suppressive function of T_{Reg} cells isolated from both the peripheral blood and the synovium found no defects in suppression[76–78]. However, in later studies Ehrenstein *et al.*[80] identified a focal defect in T_{Reg} cell function in rheumatoid arthritis with respect to the cells' ability to suppress the production of IFNγ and TNF in co-culture assays. They further established that this defect is intrinsic to the T_{Reg} cells of subjects with rheumatoid arthritis. Subsequent studies by this group have shown a defect in cytotoxic T lymphocyte antigen 4 (CTLA4)-mediated inhibition of T cell receptor signalling in T_{Reg} cells from patients with rheumatoid arthritis. This defect can be reversed by overexpression of CTLA4 in these T_{Reg} cells[81].

Resistance of effector T cells to suppression has not been tested exhaustively in rheumatoid arthritis and was not the reason for the impaired suppression observed by Ehrenstein *et al.*[80]. However, it has been shown that synovial macrophages may influence the responsiveness of effector T cells to T_{Reg} cells through their increased expression of MHC class II molecules and CD86 and increased production of TNF, IL-6 and IL-7, thereby altering the cytokine milieu and the stimulatory conditions in which suppression occurs in the joint[82].

In addition to being able to sample the target tissue, studies of rheumatoid arthritis benefit from the existence of well-established biological therapies, allowing the impact of these therapies on T_{Reg} cell number and function to be studied. This has been done in the context of the TNF-specific agent infliximab (Remicade; Centocor/Merck). Patients treated with this biological therapy were found to have an increase in the number of peripheral T_{Reg} cells, and this correlated with changes in the level of C-reactive protein, a marker of disease activity and inflammation[80]. This increase in T_{Reg} cells was not a result of expansion of the natural T_{Reg} cell population but was due to the induction of TGFβ-producing T_{Reg} cells[80].

In summary, defects in immune regulation in rheumatoid arthritis do occur and are probably due to both T_{Reg} cell-intrinsic defects and the inflammatory milieu that is present in the rheumatoid joint.

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T_{Reg} cells and IBD

The term IBD refers to two diseases — Crohn's disease and ulcerative colitis — that are distinguished by their underlying pathology. Despite pathological differences, both diseases are thought to be T cell-driven diseases and to result from a loss of immune tolerance in the gut. T_{Reg} cells have a central role in the maintenance of tolerance in the gut, which is exemplified by the wasting disease and gastritis that develop in mice lacking T_{Reg} cells and by the reversal of disease by adoptive transfer of T_{Reg} cells (reviewed in REF. [83]). A role for T_{Reg} cells in the regulation of inflammatory disease of the gut in humans is further supported by the finding that individuals with IPEX develop severe bowel inflammation as a component of their illness[13].

Studies of T_{Reg} cells in ulcerative colitis and Crohn's disease have also benefited from the ability to examine the number and function of T_{Reg} cells not only in the peripheral blood but also in the target organ. Studies of peripheral blood T_{Reg} cell numbers have given mixed results. Saruta *et al.*[84] described an increase in $CD4^+CD25^+FOXP3^+$ T cells among individuals with Crohn's disease, as also described by Takahashi *et al.*[85]. However, the same study[85] described an inverse correlation between $CD4^+CD25^+$ cells and disease activity among patients with ulcerative colitis. Maul *et al.*[86] found that the number of $CD4^+CD25^{hi}FOXP3^+$ T cells was lower in patients with active IBD (a combination of subjects with Crohn's disease and ulcerative colitis were studied) and higher in patients with inactive disease. More recent studies have described a decrease in T_{Reg} cells in subjects with active ulcerative colitis when subjects with irritable bowel syndrome were used as controls[87]. Furthermore, a relative and absolute decrease in T_{Reg} cell numbers was also described by Eastaff-Leung *et al.*[88].

Despite these differences in results from studies of the peripheral blood, observations in the gut consistently show an increase in the percentage of $FOXP3^+$ cells in inflamed lamina propria and in mesenteric lymph nodes, particularly in and near inflamed tissue[86–91]. However, the increase in T_{Reg} cells found in patients with IBD raises the question of how many T_{Reg} cells are sufficient to control inflammation. To address this question, Maul *et al.* [86] compared colonic biopsies of inflamed tissue from subjects with ulcerative colitis, Crohn's disease, diverticulitis and infectious enteritis and found that T_{Reg} cells were similarly increased in all of these inflammatory diseases. Similar findings were reported by Uhlig *et al.*[90], indicating that in IBD the increase in T_{Reg} cells seems to be similar to that accompanying all types of inflammation.

In addition to defining T_{Reg} cell numbers in the periphery and the colon, several studies have looked at the impact of therapy on T_{Reg} cell numbers. An increase in $CD4^+CD25^+$ T cells was seen in subjects with ulcerative colitis after standard treatment, correlating with their disease activity level[85], whereas T_{Reg} cell numbers were decreased among patients with Crohn's disease who were treated with thiopurines (purine antimetabolites that are widely used in the treatment of autoimmune disorders)[84]. The results from studies of infliximab treatment have been mixed: no impact of the therapy on T_{Reg} cell number was reported by one group[92], whereas two groups described an increase in T_{Reg} cells in the peripheral blood and lamina propria with therapy[87,93].

The functional studies of the T_{Reg} cells isolated from the peripheral blood, mesenteric lymph nodes or lamina propria of individuals with IBD all show that the suppression by these T_{Reg} cells is similar to that achieved by T_{Reg} cells from control individuals[84,86,89,94–97]. Although limited in scope, three studies have examined the question of the responsiveness of effector T cells in IBD. No defect was found by two groups[89,94], whereas Fantini *et al.*[98] found effector T cells to be resistant to suppression in Crohn's disease. The limitation of

each of these studies of T_{Reg} cell function is the inability to examine all of the factors that may influence their function in the lamina propria, including the cytokine milieu and the character of the APCs.

T_{Reg} cells and psoriasis

Psoriasis is a skin disorder that is characterized by erythematous scaling plaques, which are the result of inflammatory infiltrates. Psoriasis is thought to be a T cell-mediated disease of autoimmune origin, based on histological findings[99], mouse models[100] and the therapeutic efficacy of TNF-targeted therapies.

The importance of T_{Reg} cells in this disease has been examined in the peripheral blood and the inflamed skin of patients. Unlike in IBD, the number of T_{Reg} cells (defined by expression of FOXP3) in the peripheral blood of individuals with psoriasis is increased, and this increase is positively correlated with the disease activity index[101]. $CD4^+CD25^+FOXP3^+$ T_{Reg} cells are also present in psoriatic lesions[102] and, similar to the peripheral blood, are higher in lesional skin biopsies than in control or uninvolved skin biopsies[101,103]. However, an analysis by Chen *et al.*[104] found that a relative imbalance favouring effector T cells was present in both the peripheral blood and psoriatic skin lesions. Additional studies of T_{Reg} cells in patients treated with infliximab showed that T_{Reg} cell numbers were increased[105] and a more diverse T cell receptor repertoire was present in the T_{Reg} cell population[106].

The functional capacity of T_{Reg} cells in both the peripheral blood and lesional skin of patients with psoriasis is impaired with respect to their ability to suppress both autologous and control effector T cells. In addition, the effector T cells of patients with psoriasis have an enhanced proliferative capacity compared with control cells[107]. Goodman *et al.*[108] have extended these findings by identifying a mechanism that may contribute to this failure in regulation. Levels of IL-6 are increased in lesional skin, and both the effector T cell and T_{Reg} cell populations located in the skin have increased cell surface expression of the IL-6 receptor. Furthermore, IL-6-specific antibody can reverse the impairment in suppression that is observed in co-cultures of T_{Reg} cells and effector T cells from patients with psoriasis. IL-6 is known to enhance the resistance of effector T cells to T_{Reg} cell-mediated suppression[109], but Goodman *et al.* speculate that it may also inhibit T_{Reg} cell function. These studies of T_{Reg} cell function raise two potential causes of impaired T_{Reg} cell-mediated suppression in psoriasis: impaired T_{Reg} cell function and resistance of effector T cells to suppression (in part due to increased production of IL-6 at the site of inflammation, and potentially due to an increased capacity to respond to IL-6 owing to upregulated receptor expression).

Conclusion and implications

It is now clear from studies of animal models of autoimmunity that defects in T_{Reg} cell number or function can contribute to disease and that therapies directed at these defects have the potential to prevent and also cure these diseases. This knowledge is now being applied to human autoimmune disease and a picture is emerging that also implicates defects in suppression by T_{Reg} cells in these diseases (TABLE 1). Despite the example of IPEX, there is currently no clear evidence that a global deficiency in the number of T_{Reg} cells is the source of failed regulation in the more common forms of autoimmunity. Instead, the presence of increased numbers of T_{Reg} cells in the affected tissues of patients with rheumatoid arthritis, IBD and psoriasis suggests that the reason for failed regulation in the inflamed tissue may be insufficient or defective T_{Reg} cell function due to either cell-intrinsic or cell-extrinsic factors. This hypothesis is supported by the predominance of studies that find an impairment in the suppressive capacity of T_{Reg} cells from individuals with autoimmune disease, and also by our expanding understanding of how the cytokine milieu

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and local APCs may modify T_{Reg} cell function or contribute to a resistance of the pathogenic T cell populations to suppression. Such involvement of the local environment has been implicated in the pathogenesis of type 1 diabetes, SLE and possibly psoriasis.

Animal models suggest that an increase in T_{Reg} cell number at the site of inflammation is likely to be therapeutic in autoimmunity. This could be achieved in humans through adoptive transfer of *in vitro*-expanded autologous T_{Reg} cells or by the use of agents that promote T_{Reg} cell proliferation, survival and induction. However, these approaches will not target the site of inflammation; approaches that hold promise in this area include the use of antigen-specific T_{Reg} cells and/or T_{Reg} cells that express tissue-specific homing receptors. However, our current understanding of T_{Reg} cells in human autoimmune disease indicates that functional defects probably have the greatest impact on disease. The causes of these functional defects are multiple and include intrinsic defects in T_{Reg} cells and effector T cells, as well as extrinsic factors present at the site of inflammation. The mechanisms that underlie these defects are just being uncovered in human autoimmune disease, and an understanding of these mechanisms is likely to direct the development of new approaches for treating autoimmunity.

To achieve the goal of restoring tolerance in autoimmunity, the field must move forward in several ways. First, the character of T_{Reg} cell and effector T cell function during the course of an autoimmune disease must be defined. This will allow us to determine the point at which specific defects in regulation occur and help us to identify when an intervention would provide therapeutic benefit. Second, the mechanisms that cause impaired suppression by T_{Reg} cells in human disease must be determined. In some cases these mechanisms of impaired tolerance may be of genetic origin; it will be useful to define these mechanisms using data from genome-wide association studies, which would allow the assessment of how genetic variants in pathways related to T_{Reg} cells and effector T cells influence regulation. In addition, the identity of factors that influence not only cell number and function but also the plasticity of T_{Reg} cells is a newly emerging area of T_{Reg} cell biology that will need to be incorporated into these studies. Although the *in vitro* assays of human T_{Reg} cell function fail to completely mimic the *in vivo* milieu, they can help us to define the differences between cells obtained from healthy subjects and from individuals with autoimmune disease. The development of new assays that more closely replicate the *in vivo* environment should help to further resolve these issues in the future. Such approaches may require an assessment of the quality of T_{Reg} cell–effector T cell interactions, and this assessment can be carried out in short-term cultures and on a small scale using novel imaging or molecular approaches. Such approaches could then be applied to the identification of the mechanisms that underlie failed suppression and new targets for therapy.

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Glossary

Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: (IPEX). A disease caused by mutations in the transcription factor forkhead box P3 (FOXP3) and characterized by refractory enteritis and, in some patients, autoimmune endocrinopathies, autoimmune diabetes and thyroiditis. Unlike scurfy mice, peripheral blood mononuclear cells from patients with IPEX fail to produce cytokines after *in vitro* stimulation

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Scurfy mice A mouse strain with a spontaneous mutation in the transcription factor forkhead box P3 (FOXP3; also known as scurfin), which leads to a rapidly fatal lymphoproliferative disease, causing death by about 4 weeks of age. FOXP3-deficient mice lack regulatory T cells

Non-obese diabetic mice (NOD mice). NOD mice spontaneously develop type 1 diabetes mellitus as a result of autoreactive T cell-mediated destruction of pancreatic islet β-cells

DO11.10 RIP-mOVA mice A transgenic mouse model of type 1 diabetes in which a transgene encoding membrane-bound ovalbumin (mOVA) is expressed in the pancreas under the control of the rat insulin promoter (RIP) and therefore acts as a self antigen. Co-expression of a transgenic T cell receptor (DO11.10) in these mice leads to the development of spontaneous diabetes

Experimental autoimmune encephalomyelitis (EAE). An experimental mouse model of multiple sclerosis that is induced in susceptible animals by immunization with central nervous system antigens. EAE is an autoimmune disease that is mediated by $CD4^+$ T helper 1 (T_H1) cells and interleukin-17-producing T_H17 cells that are reactive to components of the myelin sheath. The cells infiltrate the nervous parenchyma, release pro-inflammatory cytokines and chemokines, promote leukocyte infiltration and contribute to demyelination

MRL–lpr mice A mouse strain that spontaneously develops glomerulonephritis and other symptoms of systemic lupus erythematosus. The lpr mutation causes a defect in CD95 (also known as FAS), preventing apoptosis of activated lymphocytes. The MRL strain contributes disease-associated mutations that have yet to be identified

Crohn's disease A form of chronic inflammatory bowel disease that can affect the entire gastrointestinal tract but is most common in the colon and terminal ileum. It is characterized by transmural inflammation, strictures and granuloma formation and is thought to result from an abnormal T cell-mediated response to commensal bacteria

Ulcerative colitis A mucosal inflammation involving the rectum and extending for a variable distance along the colon

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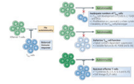

Figure 1. Causes of impaired T_{Reg} cell-mediated suppression in autoimmunity

Autoimmunity can result from a loss of regulation of autoreactive T cells. Failures of regulatory T (T_{Reg}) cell-mediated regulation include: inadequate numbers of T_{Reg} cells owing to their inadequate development, proliferation or survival; defects in T_{Reg} cell function that is intrinsic to T_{Reg} cells; and resistance of pathogenic effector T cells to suppression by T_{Reg} cells owing to factors that are intrinsic to the effector cells or factors that are present in the inflammatory milieu and that support effector T cell resistance. DC, dendritic cell; IL, interleukin; TGFβ, transforming growth factor-β; T_H17, T helper 17.

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Table 1

Overview of T_{Reg} cells in autoimmunity

Disease	T_{Reg} cell number (percentage of $CD4^+CD25^{hi}$ or $CD4^+CD25^+FoXP3^+$ cells)		T_{Reg} cell function	Effector T cell resistance	Response to therapy
	Peripheral blood	**Tissue**			
Type 1 diabetes	Normal	ND	Decreased	Increased	ND
Multiple sclerosis	Normal; altered subsets of T_{Reg} cells	Increased in the CNS	Decreased	Normal	Increased T_{Reg} cell numbers with IFNβ therapy[53]
Systemic lupus erythematosus	Decreased	ND	Decreased	Increased	Increased T_{Reg} cell numbers with corticosteroids
Rheumatoid arthritis	Increased	Increased in the synovial fluid of active disease	Decreased	Normal	Increased T_{Reg} cell numbers with infliximab therapy correlating with change in C-reactive protein
Inflammatory bowel disease	Decreased in active ulcerative colitis; normal in Crohn's disease	Increased in the lamina propria and mesenteric lymph nodes	Normal	Normal	ND
Psoriasis	Increased	Increased in the skin	Decreased	Increased	ND

CNS, central nervous system; FOXP3, forkhead box P3; IFNβ, interferon-β; ND, not determined; T_{Reg}, regulatory T.