Regulatory T cells: A review

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ABSTRACT
Regulatory T cells (Tregs) play a pivotal role in the homeostasis of the immune system and in the modulation of the immune response. Tregs have emerged as key players in the development and maintenance of peripheral immune tolerance. Broadly speaking, CD4+ T cells possessing the ability to suppress immune responses can be divided into two types: naturally occurring (nTreg) and inducible (iTreg) or adaptive regulatory cells. Naturally occurring thymus-derived CD4+CD25+ Tregs are a subset of T cells which have immunosuppressive properties and are 5%–10% of the total peripheral CD4+ T cells. In normal conditions, Tregs regulate ongoing immune responses and prevent autoimmunity. Imbalanced function or number of these cells, either enhanced or decreased, might lead to tumour development and autoimmunity, respectively. These cells thus play a major role in autoimmune diseases, transplantation tolerance, infectious diseases, allergic disease and tumour immunity. These natural properties make Tregs attractive tools for novel immunotherapeutic approaches. The in vivo manipulation or depletion of Tregs may help devise effective immunotherapy for patients with cancer, autoimmunity, graft-versus-host disease, infectious diseases and allergic diseases. It is crucial to understand the biology of Tregs before attempting therapies, including (i) the injection of expanded Tregs to cure autoimmune disease or prevent graft-versus-host disease or (ii) the depletion or inhibition of Tregs in cancer therapy. Recent findings in murine models and studies in humans have opened new avenues to study the biology of Tregs and their therapeutic potential. This overview provides a framework for integrating these concepts of basic and translational research.


INTRODUCTION
Regulatory T cells (Tregs) play a critical role in the modulation of immune response and have emerged as key players in the development and maintenance of peripheral immune tolerance. This review presents the current perspective on new developments in the biology and function, role and application of Tregs relevant to a variety of clinical conditions and avenues for their therapeutic modulation.

ORIGIN AND DISCOVERY
As self-reactive T and B cells can be demonstrated in the peripheral blood of the host (as a result of breakdown of clonal deletion process or central tolerance) and the self-reactive cells can, under certain circumstances, be activated to cause tissue damage in autoimmunity, Gershon and Kondo in 1971 suggested that there was probably a second regulatory mechanism and that a subset of suppressor T cells should exist in the host to maintain tolerance to self-antigens in the periphery. Due to conflicting results, the concept of T cell suppression fell into disrepute and research waned till the late 1980s. It took more than two decades to overcome the lack of evidence for phenotypic or functional markers of so-called suppressor T cells, as suggested by Gershon, and identify a subset of CD4+ T cells co-expressing CD25 that downregulate the activation and expansion of self-reactive T cells.

There was a resurgence of interest in this field with the seminal observations of the Sakaguchi group in the mid-1990s in what is now termed as Tregs. They demonstrated that a minor population of CD4+ T cells that co-expressed the CD25 antigen (the IL-2Rα-chain) function as Tregs in the normal adult and that CD4+CD25+ T cells exhibit an enormously suppressive activity and prevent autoimmunity in a murine model. This and other data collectively suggested the existence of a thymically produced suppressive T cell population, which was responsible for the establishment and maintenance of peripheral self-tolerance.

The understanding of the biology of Tregs took a leap forward in 2003 following the delineation of the functional and developmental role played by the transcription repressor—the X-chromosome-encoded forkhead-winged-helix transcription factor, FoxP3, as the key player in the biology of CD4+CD25+ Tregs and as a specific lineage marker and master regulator of Tregs. FoxP3 protein remains the best and the most specific marker of Tregs cells to date. Studies in the mouse show that FoxP3-deficient animals lack Tregs, whereas over-expression of the FoxP3 protein leads to profound immune suppression. Young men with the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) or a mutant mouse strain, scurfy, both succumb to similar autoimmune and inflammatory diseases due to uncontrolled activation of CD4+ T cells. Both IPEX patients and scurfy mice have mutations in a common gene, FoxP3.

Subsets of Tregs
Currently, various subsets of Tregs have been identified based on their expression of cell surface markers, production of cytokines
and mechanisms of action (Figs 1 and 2). They are broadly divided into two subsets, naturally occurring (nTreg) and induced (iTreg), based on their ontogeny and mode of action (Figs 1 and 3). In brief, Tregs classified on the basis of origin, generation and mechanism of action, appear to have complementary and overlapping functions in the control of immune response. In peripheral T cell immunoregulation, the subset of nTregs can work in synchrony with iTregs to control the activation and function of adaptive immune response. nTreg suppression is primarily cytokine-independent while that of iTreg is primarily cytokine-dependent. nTregs, which are generated in the thymus, are characterized by the constitutive expression of intracellular cytokine-dependent. nTregs, which are generated in the thymus, primarily cytokine-independent while that of iTreg is primarily cytokine-dependent. nTregs, which are generated in the thymus, are characterized by the constitutive expression of intracellular cytokine-dependent.

**Phenotype or marker molecules of Tregs**

Tregs were originally defined on the basis of constitutive expression of surface CD4 antigen and surface CD25 antigen (IL-2 receptor \( \alpha \)-chain) at high density. Early studies lacked the incorporation of FoxP3, which has been recognized as a master regulator and lineage-specification factor for Tregs. More recently, studies have shown that reciprocal expression of the IL-7 receptor (CD127) on FoxP3+ Tregs is a more specific way to quantify Tregs. nTregs constitute approximately 5%–10% of the peripheral CD4+ T cell population in normal naive mice and healthy humans and are characterized by the constitutive expression of CD25 (IL-2 receptor \( \alpha \)-chain) and low expression levels of CD45RB.

The CD4+CD25+ phenotype for Tregs has been insufficient to define them as CD25, is not T cell restricted and cannot be used to distinguish Tregs from effector T cells (Teffs). While the murine CD4+CD25+ population is highly enriched in Tregs, in humans CD25+ cells contain both Treg and Teff populations. To obtain enriched Tregs with little Teff contamination, it is necessary to gate on the CD4+CD25+ population that has regulatory activity. This population accounts for only ~1%–3% of human CD4+CD25+ T cells. The CD4+CD25+CD127low population contains approximately 80% of the FoxP3+ cells and is significantly larger than the CD4+CD25+ population. Overall, the available data indicate that FoxP3 identifies a broader Treg population than that defined by CD4+CD25+ or CD4+CD25+CD127low expression alone. A definition of Tregs by combining CD127 and FoxP3 has the advantage of including not only Tregs expressing high levels of CD25 but also Tregs with low CD25 expression and excluding at the same time activated conventional T cells. While a strong correlation between FoxP3 and CD25 expression in the resting CD4+ T cell population has been reported, low levels of FoxP3 are detectable in CD25–CD4+ T cells. Thus, it seems that FoxP3 expression too, in humans, might not be confined to Tregs.

Other cell-surface markers associated with the phenotype and function of Tregs are CTLA-4 (or CD152), GITR, CD62 ligand (CD62L), TGF-β1, IL-10, lymphocyte activation gene-3 (LAG-3), integrin αEβ7 (CD103), neuropilin-1 (Nrp1) and OX40 (CD134).

**Function**

T-cell-mediated suppression by Tregs is a key mechanism for preserving self-tolerance, not only to autoantigens but also to foreign antigens in mice and humans, indicating that Tregs play a pivotal role in the homeostasis of the immune system. Tregs have thus emerged as key players in the induction and maintenance of immunological tolerance. Tregs provide protection from autoimmune disease, graft versus host disease (GVHD), transplant rejection and overwhelming tissue destruction during infections. Conversely, high Treg numbers enable cancer cells to evade the host immune response.

**Table I. Different subsets of regulatory T cells (Tregs)**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Phenotype</th>
<th>Suggested immunosuppressive mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4+</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural regulatory T cells (nTreg)</td>
<td>CD25+FoxP3+45+RO+CTLA-4 GITR+CD134+CD62L+CD103+ lymphocyte activation gene-3+CD127loCD26+</td>
<td>Cell-to-cell contact-dependent in vivo (CTLA-4) cell-to-cell contact and cytokine-dependent in vivo (IL-10 and TGF-β)</td>
</tr>
<tr>
<td>Inducible regulatory T cells (iTreg)</td>
<td>CD25+FoxP3+45+RO+CTLA-4+CD127loCD26+</td>
<td></td>
</tr>
<tr>
<td>Th3</td>
<td>CD25±FoxP3±45+RO+CTLA-4+</td>
<td>Cytokine-mediated (TGF-β production&gt;&gt;IL-10) Cell-to-cell contact, cytokine-mediated (IL-10&gt;&gt;TGF-β, IL-5, IFN-γ production)</td>
</tr>
<tr>
<td>Tr1</td>
<td>CD25±FoxP3±45+RO+CTLA-4-</td>
<td>Cytokine-mediated (IL-10 and TGF-β production)</td>
</tr>
<tr>
<td>TGF-β1/IL-10 double positive Treg</td>
<td>CD25-FoxP3-</td>
<td></td>
</tr>
<tr>
<td><strong>CD8+</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T suppressor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naturally occurring</td>
<td>FoxP3+45+RO+CD25+CTLA-4 GITR+</td>
<td>Cell-to-cell contact-dependent (CTLA-4) Cytokine-mediated (TGF-β production)</td>
</tr>
<tr>
<td>Non-antigen specific</td>
<td>CD28-FoxP3+CD56-</td>
<td>Cytokine-mediated (IL-10 production) Cell-to-cell contact</td>
</tr>
<tr>
<td>Inducible p8 T cells</td>
<td>CD25+FoxP3+CD28+GITR+CTLA-4+</td>
<td>Cell-to-cell contact, cytokine-mediated</td>
</tr>
<tr>
<td><strong>CD4-CD8-αβ T cells</strong></td>
<td>CD25+30+45+69+LFA-1+CTLA-4+</td>
<td>Cell-to-cell contact, produce IFN-γ and TNF-α</td>
</tr>
<tr>
<td><strong>Natural killer T cells</strong></td>
<td>CD161</td>
<td>Cytokine-mediated, produce both Th-1 (IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-10, IL-13)</td>
</tr>
</tbody>
</table>

CTLA-4 cytotoxic T lymphocyte-associated antigen-4, GITR glucocorticoid-induced TNFR-related protein, Tr1 regulatory T cells type 1, Th3 T helper 3.
REGULATORY T CELLS IN HUMAN DISEASE

Tumour immunity

Cancer develops as a part of ‘self’ and many tumour-associated antigens are self-antigens recognized by autologous T cells. Therefore, the mechanisms that maintain central or peripheral immunological tolerance to self-antigens may inhibit the generation of effective tumour immunity. nTregs impede immune surveillance against autologous tumour cells and may inhibit the generation, activation, proliferation and effector function of tumour-infiltrating T cells.

Expansion of CD4+CD25+ Tregs within the tumour microenvironment, draining the lymph nodes and peripheral blood has so far been accepted as a hallmark of cancer. In the context of cancer, the importance of Tregs lies in the fact that increased numbers may favour tumour development or growth and influence the course of the disease. Augmented Treg frequencies have been linked to tumour stage, prognosis and survival.

Non-specific depletion of CD4+ T cells can lead to the induction of efficient antitumour immunity. Onizuka et al. in 1999 were the first to suggest that Tregs played an important role in inhibiting tumour immunity. They showed that reduction of CD4+CD25+ Tregs by anti-CD25 monoclonal antibody treatment resulted in rejection of immunogenic tumours in none or less responding animals. Various murine tumour models exploring the suppressive function of Tregs in tumours such as fibrosarcoma, melanoma, colon carcinoma, pancreatic cancer and prostate dysplasia have been used by different workers. The relatively early induction of Tregs in tumour development implies that their presence would precede the time of diagnosis in most patients. Tregs are attracted to the site of the tumour and one of the possible mechanisms is shown in Fig. 4. The in vivo manipulation or depletion of Tregs may help in devising effective immunotherapy for cancer patients and represent a novel way of provoking tumour immunity. However, further work is needed to understand how enrichment of Tregs occurs in cancer patients and whether accumulation or preferential induction of tumour-specific Tregs plays a role during tumour progression.

Solid cancers

Numerous investigators have reported the important role played by Tregs in the immune response and prognosis of solid tumours. Increased percentages of Tregs were first reported in tumour-infiltrating lymphocytes in non-small-cell lung cancer and ovarian cancer. Following this initial observation, further studies concluded that Tregs are increased not only in the tumour microenvironment of patients with invasive breast or pancreatic
survival. Subsequently, Treg frequencies were also found to accumulate during progression and are associated with decreased immunity in ovarian cancer, contributing to tumour growth, and demonstrated that Tregs suppress tumour-specific T cell-mediated increased in squamous cell carcinoma of the head and neck, hepatocellular carcinoma and mesotheliomas. Hence, the cancers but also in the peripheral blood, suggesting that the increase of Tregs is a generalized phenomenon. In malignant melanoma, patients have a two-fold increased frequency of Tregs in metastatic lymph nodes compared with tumour-free nodes. In patients with gastric carcinoma, the frequency of peripheral Tregs is inversely correlated with their prognosis. Curative resections in patients with gastric carcinoma, the frequency of peripheral Tregs is inversely correlated with their prognosis. Curative resections with optimal TCR stimulation, CD28 signalling, L-2,4,6,7,8 TCR stimulation and co-stimulation. TCR stimulation of high-to-medium affinity is required for nTreg activation while suboptimal stimulation is required in case of iTregs. In addition to TCR, CD28 co-stimulation seems to play an important role in the differentiation of Tregs. Yet these signals may not be sufficient for FoxP3 upregulation and other signals may be required, e.g. IL-2 and, to a lesser degree, two other common γ-chain (γc) cytokines, IL-7 and IL-15 (reproduced with permission from Lourenço and La Cava, Autoimmunity 2011;44:33–42).

Haematological malignancies
In comparison to the interest in number of Tregs in solid tumours, developments in the role of Tregs in haematological malignancies took a while to occur, and are relatively recent. A variety of methods have been devised to study Tregs in haematological malignancies, the most predominant of these being the use of flow cytometry.

The first study in this field demonstrated large populations of both IL-10-secreting Tr1 and CD4+CD25+ Tregs in infiltrating lymphocytes and peripheral blood mononuclear cells in Hodgkin lymphoma. In patients with B-cell chronic lymphocytic leukaemia, a stage-dependent increase of CD4+CD25 highFox P3+CTLA4+GITR+Tregs with frequencies reducing after fludarabine therapy has been established. However, patients with decreased Tregs are prone to autoimmune disease and increased frequency of autoimmune haemolytic anaemia (AIHA), and thrombocytopenia is linked to fludarabine therapy.

Similar studies have reported an expanded pool of Tregs in B-cell non-Hodgkin lymphomas, acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML). Increased frequencies of CD4+CD25 highFoxP3+ Tregs have also been shown in multiple myeloma as well as its premalignant precursor monoclonal gammopathy of undetermined significance. Data suggest that peripheral expansion of Tregs occurs frequently in high-risk myelodysplastic syndrome as well as during disease progression. Taken together, these data establish the concept of increased Tregs in haematological malignancies. However, some of the early studies need to be validated by using more specific markers such as FoxP3 as well as more sophisticated and standardized functional assays.
**Transplantation tolerance**

The finding that depletion of CD4+CD25+ Tregs from normal mice enhances graft rejection suggests that these cells are involved in transplantation tolerance. On the other hand, an increase in the absolute number of Tregs may lead to significant prolongation of graft survival. Several studies indicate that Tregs not only play a role in solid organ transplantation but also seem to inhibit GVHD after stem cell transplantation (SCT). Murine studies have established an important role for Tregs in the suppression of GVHD without compromising a graft-versus-leukaemia (GVL) effect. Clinical studies are ongoing on the feasibility and clinical consequences of adoptive transfer of this cellular population after allogeneic SCT (alloSCT). Strategies that augment a GVL effect without increasing the risk of GVHD are required to improve the outcome after alloSCT.

In humans, prospective studies have shown that Treg frequencies were significantly lower in patients with acute GVHD (aGVHD) than in those who did not have aGVHD or who underwent autologous SCT. Moreover, there was an inverse linear correlation between frequencies of Tregs and severity of aGVHD. Similarly, patients with chronic GVHD (cGVHD) had a lower frequency of Tregs as compared to those who had no cGVHD. Increased Treg content in the graft was found to be associated with less aGVHD in alloSCT after myeloablative conditioning regimens in some studies, but not in all. The reason for these apparent disparate results is not known. The Stanford group has demonstrated that Tregs suppress early expansion of allogeneic donor T cells and CD25 expression, decreasing GVHD but without abrogating the GVL effect.

Recent studies have reported that the CpG island associated with the promoter of the FoxP3 gene is hypermethylated in CD4+CD25+ cells and that administration of DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (AZA) upregulates CD4+CD25+ cells and that administration of DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (AZA) upregulates FoxP3 expression of FoxP3, resulting in an in vitro expansion of Tregs. In murine transplantation models, administration of AZA after transplantation results in expansion of Tregs and a reduction in the incidence of aGVHD without apparent abrogation of a GVL effect.

**Autoimmunity**

Sakaguchi et al. first reported in 1995 that the transfer of CD4+CD25− T cells into athymic nude mice induces various organ-specific autoimmune diseases, whereas the co-transfer of a small number of CD4+CD25+ T cells could completely prevent the onset of autoimmunity. The IPEX syndrome alluded to previously made it apparent that a deficiency of nTregs caused by mutations in the Foxp3 gene resulted in autoimmune lesions in multiple tissues that manifest early in life. Apart from this mutation, humans expressing a defective form of the transcription factor autoimmune regulator (AIRE) develop multorgan autoimmune disease. Mutations of the AIRE gene are seen in an autosomal monogenic disease—autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophies (APECED). It has been assumed that mutations within the AIRE gene may lead to insufficient negative selection of self-reactive T cell clones and defective generation of thymus-derived Tregs.

Decreased numbers and functional activity of CD4+CD25+ FoxP3+ T cells (Tregs) are associated with impaired immune homeostasis and an increased susceptibility to development of various autoimmune diseases. Several studies in experimental animal as well as human autoimmune disorders, such as multiple sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type 1 diabetes, psoriasis, myasthenia gravis, Kawasaki disease, autoimmune polyglanudular syndrome type II and autoimmune lymphoproliferative syndrome have reported a decrease in circulating CD4+CD25+ Tregs compared to that in healthy individuals. It is likely that most autoimmune diseases have a common mechanism. A question which needs to be answered is whether the onset of autoimmune diseases is determined by a dysfunction/deficiency of nTregs by an imbalance between self-reactive T cells and nTregs. CD4+CD25+ Tregs from patients with active RA are both phenotypically and functionally abnormal. Increased number of Tregs has been detected in the synovial fluid (SF) of patients with active RA, suggesting migration of Tregs from the peripheral blood to the site of inflammation. Moreover, Tregs of patients with RA showed diminished in vitro activity to suppress the production of interferon-γ (IFN-γ) and TNF-α by the CD4+CD25− Teffs which may contribute to the ongoing inflammation. Several cytokines can influence the activity and number of Tregs, negatively or positively, in physiological and pathological conditions. IL-6 appears critical in the inhibition of differentiation of FoxP3+ Tregs and the suppressive activity of Tregs. IL-6 is also found in high levels in SF from the joints of patients with active RA which renders responder T cells resistant to CD4+CD25+ Treg suppression.

TNF has been described to inhibit the suppressive function of both nTregs and TGF-β iTregs. TNF-mediated inhibition of suppressive function is related to a decrease in FoxP3 mRNA and protein expression by Tregs. Notably, CD4+CD25high Tregs isolated from patients with active RA expressed reduced levels of FoxP3 mRNA and protein. Treatment with anti-TNF antibody (infliximab) increased FoxP3 mRNA and protein expression by CD4+CD25high Tregs and restored their suppressive function. CD4+CD25high Tregs isolated from patients with active SLE expressed reduced levels of FoxP3 mRNA and protein and poorly suppressed the in vitro proliferation and cytokine secretion of CD4+ Teffs. In vitro activation of CD4+CD25high Tregs from these patients restored their suppressive function. Overproduction of TNF may also contribute to the defective function of Tregs in patients with active SLE.

An autoimmune pathophysiology has also been postulated for several haematological disorders. In vitro studies in aplastic anaemia (AA), pure red cell aplasia, AIHA and primary immune thrombocytopenia (ITP) support the view that the disease mechanism may in part be mediated by self-reactive T cells. In a murine model of AIHA, depletion of CD4+CD25+ Tregs resulted in an increased incidence of AIHA in C57/B16 mice. In patients with ITP, naturally occurring CD4+CD25+ Tregs are both functionally impaired and reduced in number. It has been found that in patients with ITP the number of CD4+CD25+ T cells was significantly lower in the severe phase. However, the number of those cells increased in the patients in the complete remission phase, especially after a splenectomy.

A study undertaken to investigate the role of Tregs in preventing ITP in a murine model revealed that Treg-deficient mice prepared by inoculation of Treg-depleted CD4+CD25− T cells isolated from BALB/c mice into syngeneic nude mice intravenously spontaneously developed thrombocytopenia. Simultaneous transfer of Tregs completely prevented the onset of thrombocytopenia, but transfer of Tregs after the onset of thrombocytopenia had no apparent effect. These results indicate that Tregs play a critical role in preventing murine autoantibody-mediated thrombocytopenia by engaging CTLA-4.
In yet another study, it was reported that thrombopoietic agents in patients with ITP have profound effects in restoring immune tolerance and improving Treg activity.64

Two studies have shown that Tregs are significantly lower at presentation in almost all patients with AA than in controls.67,68 Activated- and resting-Tregs were reduced in AA. Moreover, a functional impairment of Tregs was observed.68 Treg defects are also now implicated in autoimmune marrow failure.67

A study strongly suggests that the deficiency of Tregs might play an important role in the immunopathophysiology of autoimmune neutropenia in children.69

In an elegant study, it has been shown that abnormalities in Tregs may contribute to the pathogenesis of autoimmune complications associated with Wiscott–Aldrich syndrome.70

**Infectious diseases**

Tregs have a role to play in infectious diseases by blunting overactive and damaging immune responses to pathogens. Another but not necessarily exclusive role of Tregs might be to moderate the degree of inflammation and damage in a tissue during the course of any early infection. In infectious diseases, however, the situation is ambivalent, i.e. sometimes Tregs are useful and at other times not.

During infection, Tregs seem to play a key role in preventing immunopathological processes and limiting damage to the host due to immune responses to the pathogen. Tregs can also be exploited by the pathogen to subvert protective immune responses and thereby, prolong survival in the host. This is a feature of pathogens that cause chronic infection. In many of these infections, a failure to clear the infection can be attributed directly to potent Treg responses. Also in mouse models, depletion or inactivation of Tregs can enhance clearance of the pathogen.

Tregs are intricately involved in the immune response to many parasitic infections. During malarial infection, increased numbers of CD4+CD25+FoxP3+ T cells have been found in both human and murine models. Higher number of Tregs are associated with increased parasite load and development of human infection caused by *Plasmodium falciparum*.71

The CD4+ Treg population has also been found to be significantly higher in several filarial infections. The human filarial parasite has the ability to stimulate Treg activity, providing further support for the hypothesis that these cells play an important role in the infective process. Adult parasites also induce FoxP3 expression. A study indicated that, in the mouse at least, filarial parasites expand the frequency and activity of FoxP3-expressing T cells at the site of infection, and that these cells have functional in vitro suppressive capacity indicative of Tregs.72

Tregs are also involved in immunoregulatory mechanisms in parasitic infections such as trypanosomiasis, toxoplasmosis and schistosomiasis. Visceral leishmaniasis (VL) represents a parasitic disease that has been shown not to induce expansion of nTregs. In a study among patients with Kala-azar, frequencies of FoxP3+ cells in patient with VL before and after treatment did not increase, neither were they elevated when compared to endemic controls. It was therefore concluded that active VL is not associated with increased frequencies of peripheral FoxP3 Tregs or accumulation at the site of infection. While active VL does not induce expansion of Treg, it has been shown in animal models that Treg is directly responsible for its reactivation.73

Several studies have reported involvement of Tregs in viral diseases as Tregs might affect the magnitude of the immune response and therefore the outcome of viral clearance. After depletion of Tregs by anti-CD25 antibody in herpes simplex virus-infected mice, increased CD4+ T cell responses, enhanced CD8+ proliferative and cytotoxic T lymphocyte (CTL) responses, and increased mucosal antibody levels were reported as compared to those in non-depleted animals. In addition, viral clearance occurred more rapidly in Treg-depleted mice. In humans with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, an increase in peripheral CD4+CD25+ Tregs has been described as compared to that in healthy individuals. Moreover, these Tregs are able to suppress HCV-specific CD8+ T cell immune responses. Besides increased levels of Tregs in patients, IL-10-producing Tr1 cells could also be isolated and cloned from patients with chronic HCV infection, but not from patients who cleared the infection. Following the demonstration of the role of Tregs in suppressing antiviral immune responses, several *in vitro* studies showed that depletion of Tregs from the peripheral blood of patients with viral infection results in increased T cell responses to HBV, HCV, cytomegalovirus (CMV), and human immuno-deficiency virus (HIV).74 While the results are clear for HBV, HCV and CMV infections, the influence of Tregs during HIV infection might be more complex.

Substantial research has been done on the role of Tregs in HIV infection. There is usually an increased frequency of Tregs in lymphoid tissues of HIV-infected persons. In HIV infection, though most studies demonstrate Treg expansion as compared with healthy controls,75 one study reported that Tregs were depleted.76 To date, it is still not clear whether Tregs are detrimental to HIV infection because they suppress HIV-specific responses, or if they are beneficial because they decrease immune activation associated with HIV infection.77

*Mycobacterium tuberculosis* (Mt) is a good example of a bacterium that causes chronic infection, where Tregs and Mtb have been implicated in pathogen persistence and protection against immune-mediated pathology. Tregs are enhanced in patients with active disease, where they impair immune responses, especially IFN-γ production by protective Th1 cells. Tregs are prominent in tubercular granulomas and pleural effusions. FoxP3+ Tregs are significantly higher in the peripheral blood of patients with active compared with those with latent tuberculosis (TB). Tregs may also render patients with latent TB susceptible to reactivation. *Ex vivo* depletion of Tregs in human peripheral blood mononuclear cells from patients with active disease enhances IFN-γ production in response to Mtb antigens.77

**Allergic diseases**

Allergy, atopic or non-atopic, is an altered immune reactivity to harmless environmental allergens to which one has previously been exposed. Allergic response to an allergen is subject to a complex regulatory control, which includes Tregs. Experiments in mouse models and humans have shown a deficiency of Tregs in allergic diseases such as asthma, allergic rhinitis, eczema and atopic dermatitis.78 Different regulatory cell types have been implicated in the prevention of atopic sensitization in mouse models and humans. In addition to nTregs, iTregs, induced in response to foreign antigens, have been demonstrated. The cytokines most commonly implicated in Treg-mediated suppression of allergic asthma are TGF-β and IL-10.79

A decreased expression of FoxP3 has been reported in allergic diseases. In humans, along with typical disease manifestations such as elevated IgE levels, eczema, insulin-dependent diabetes mellitus, intestinal inflammation, asthma and other recurrent respiratory disorders have also been associated with IPEX in
which there is a mutation of the FoxP3 gene. Although eczema is a common feature of IPEX-deficiency, there is less documentation of asthma in patients with IPEX. The most likely reason for this is that there is inherently significant uncertainty in the diagnosis of asthma in young children.  

According to the ‘hygiene hypothesis’, which was postulated to explain the increased prevalence of allergic diseases in developed countries in recent years, early childhood exposures to pathogen-associated products inversely correlates with the incidence of allergic diseases in adulthood. With the rapid progress of research in the field of Tregs in recent years, it appears that microbial infections are conducing to the development of these cells which are then able to exercise their immunosuppressive functions to dampen unwarranted immune responses against foreign or self-antigens. 

REGULATORY T CELLS AS POTENTIAL IMMUNOTHERAPY  

There are a variety of circumstances where manipulation of Tregs could prove beneficial. Studies using mouse experimental models have provided compelling evidence that Tregs can induce transplantation tolerance, prevent GVHD, cause regression of tumours and control allergy and autoimmunity.

**Tumour immunity**  
The suppressive Tregs present within the tumour microenvironment and draining lymph nodes may help mediate immune evasion. Therefore, in cancers, the general objective has been to blunt or negate the activity of Tregs so that the function of antitumour Teff can proceed more effectively. Targeting Tregs therefore provides an attractive immunotherapeutic strategy to potentially influence the suppressed immune response in patients with tumour, thereby altering and supporting conventional antitumour therapy. However, such strategies need to be undertaken cautiously as these mechanisms modify immune homeostasis which may predispose to the development of autoimmune diseases.  

The Sakaguchi group and others have described tumour models in mice where suppression of Tregs with anti-CD25 or anti-GITR monoclonal antibody appears to achieve this antitumour effect. In a study on a murine model, an agonistic anti-GITR antibody targeting Tregs has been successfully applied in tumour-bearing mice.  

A specific approach for therapeutic targeting of Tregs may be to attempt to remove Tregs by targeting the CD25 receptor using an IL-2 diphtheria toxin conjugate. Pilot studies on cancer patients indicate that this achieves Treg reduction in the peripheral blood and has led to clinical improvement in some cases. Studies suggest that the effectiveness of a cancer vaccine would be augmented by this synergistic approach. The depletion of CD25+ T cells with anti-CD25 monoclonal antibody before dendritic cell (DC)-based vaccination dramatically improved survival in mice compared with those receiving vaccination alone. A recombinant IL-2 diphtheria toxin conjugate DAB(389)IL-2 (denileukin diftitox) has been used in a study to selectively eliminate CD25-expressing Tregs from the peripheral blood mononuclear cells of patients with cancer. This strategy significantly reduced the number of Tregs present in the peripheral blood of patients with metastatic renal cell carcinoma. In yet another study, analysis of circulating Tregs in patients with metastatic prostate cancer vaccinated with PSA-TRICOM vaccine showed a decrease in the suppressive function of Tregs in the post- versus pre-vaccination scenario and its possible association with improved overall survival.  

There are various possible ways by which the frequencies or suppressive functions of Tregs can be downregulated. Depletion of Tregs with anti-CD25 agents has been the most common approach. Pharmacological means to deplete Tregs include reagents such as denileukin diftitox (anti-CD25 Ab), low-dose cyclophosphamide, fludarabine, ipilimumab (anti-CTLA-4, a fully human monoclonal antibody), anti-GITR mAb, anti-OX40, anti-TGF, anti-toll-like receptor and 1-methyl-d-tryptophan (D-1MT). Denileukin diftitox (Ontak) has been approved by the US Food and Drug Administration (FDA) to treat cutaneous T cell leukemia/lymphoma for the past 3 years with no reported increased incidence of autoimmune disorders. Additional reports confirm that denileukin diftitox depletes the number of Tregs and the suppression mediated by the CD4+CD25+ T cell population and improves immunity in individuals with renal cell carcinoma and melanoma.  

While ipilimumab has been approved for the treatment of unresectable or metastatic melanoma; anti-TGF is being used in trials in myelofibrosis, mesothelioma and systemic sclerosis; anti-OX40 is being used in trials in prostate cancer and asthma; and D-1MT is being used in trials in refractory solid tumours. A phase 1/2 trial evaluating ipilimumab in patients with follicular lymphoma is ongoing. Low-dose cyclophosphamide has been successfully tested in murine cancer models to cause immunologically mediated regression of immunogenic lymphomas.  

Cyclophosphamide depletes Tregs and boosts efficacy of a DC vaccine in mouse models for melanoma or colon carcinoma. This immunostimulatory agent, thus, might be successfully integrated into chemoimmunotherapy.  

The mammalian target of rapamycin (mTOR) inhibitor everolimus in addition to inhibiting immune responses enhances Treg conversion by several distinct pathways. The converted Tregs can be further expanded by injection of IL-2. The combined use of everolimus and IL-2/IL-2αβ complexes in vivo makes it feasible to achieve highly effective antigen-driven conversion of naive T cells into Tregs and their expansion in vivo and thereby the described protocols constitute important tools to achieve immunological tolerance by Treg vaccination. Vaccination against FoxP3 improved tumour immunity in a model for renal cell carcinoma. CpG treatment lowers FoxP3+ T cells in the lymph nodes of patients with melanoma.  

While there are limitations of currently available Treg-depletion strategies, their employment has already begun to show a translational impact. However, further studies to extend these findings are warranted.  

**Transplantation**  
Reinforcement of the function of Tregs in transplantation may mediate graft survival and this has set the stage for the potential therapeutic application of Tregs in transplant patients. In rodents, Treg depletion from the donor graft accelerates GVHD lethality, suggesting a role of endogenous donor Treg-mediated suppression during the GVHD response. Studies in murine models have shown that iTregs were much less effective in preventing GVHD than nTregs. However, recent studies in a human xenogenic GVHD model reveal comparable suppression by in vitro-expanded, polyclonally activated iTregs and ex vivo-expanded nTregs.  

Within 8 years of the first demonstration of the efficacy of Tregs in suppressing GVHD in mouse models, three clinical trials have been reported that are evaluating the safety and efficacy of Tregs in treating GVHD, all demonstrating potentially safe and reliable efficacy profiles. In these preliminary studies on humans, Tregs obtained from adult donors or umbilical cord blood were administered after transplantation to prevent GVHD.
The first-in-man trial involved two patients. The first patient had cGVHD two years after transplantation. After receiving 0.1 × 10^6/kg fluorescence-activated cell sorting (FACS) purified ex vivo-expanded Tregs from the donor, the symptoms subsided and the patient was successfully withdrawn from immunosuppression. The second patient had acute disease that progressed despite three infusions with an accumulative dose of 3 × 10^6/kg expanded donor Tregs. A larger scale phase 1 trial has recently been concluded in which it was shown that ex vivo-expanded Treg infusions from a third party could be used as supplemental GVHD prophylaxis after double umbilical cord blood transplantation. Twenty-three patients with advanced haematological malignancies were enrolled and treated with two units of umbilical cord blood as source of stem cells and T cells. Tregs were isolated using anti-CD25 immunomagnetic bead selection from third-party cord blood samples that had 4–6 matches of human leukocyte antigen (HLA) with the recipient. Tregs infused with the graft were detectable in the peripheral blood up to day 14 after transplantation. During the 1-year period after Treg infusion, the investigators observed no dose-limiting toxicities or increase in adverse events when compared with historical controls. Incidences of severe aGVHD were significantly reduced in patients who received Treg therapy.

In the third trial, freshly isolated anti-CD25 immunomagnetic bead-enriched donor Tregs without ex vivo expansion were infused 4 days before the infusion of CD3+ cells and conventional T cells from the same donors in 28 patients with high-risk haematological malignancies undergoing haploidentical transplantation. No adjunct immunosuppression was given after transplant. Patients demonstrated accelerated immune reconstitution, reduced CMV reactivation, and a lower incidence of tumour relapse and GVHD when compared with historical controls. Only 2 (7%) patients developed aGVHD. These encouraging early experiences support further investigation of the efficacy of Treg therapy in controlling GVHD and applying it in other disease settings.

There are numerous ongoing trials in AML which are testing whether Treg manipulation in AML can decrease GVHD. Strategies that augment a GVL effect without increasing the risk of GVHD are required to improve the outcome after alloSCT. Wider applications of Treg therapy are also being considered in other disease settings such as solid organ transplantation. However, several aspects of Treg biology that have particular relevance to disease settings such as solid organ transplantation. Autoimmunity, the problem is often a failure of one or more regulatory cells types to function effectively. The challenge is to find ways to boost their activity. The practical problem is to use Tregs that can interfere with ongoing autoimmune disease and cause its reversal or even resolution. Research in animal models has demonstrated that Tregs can be used to treat many autoimmune diseases such as type 1 diabetes, inflammatory bowel disease, SLE, multiple sclerosis, RA and autoimmune gastritis.

FACS-based isolation can provide high-yield CD4+CD25+CD127low Tregs for ex vivo expansion of highly pure Tregs; this protocol has been approved by the US FDA for a phase 1 safety trial in patients with type 1 diabetes (NCT01210664). CD3-specific antibodies have been used successfully in patients with autoimmune diseases, particularly type 1 diabetes. The in vivo induction of Tregs using, for example, anti-CD3 antibodies or autoantigenic peptides currently appears closer to clinical application.

Recent studies demonstrate that nTregs are unstable and dysfunctional in inflammatory conditions. The stability and function of iTregs in established autoimmune diseases, their advantage as therapeutics under these conditions and proper generation and manipulation of iTregs used for cellular therapy is yet another area of active investigation which is being explored. Immunomodulation with manipulation of Tregs may become a part of treatment of autoimmune disorders in the future.

However, a number of issues must be addressed before CD4+CD25+ Treg therapy can be considered for use in humans. These are:

1. A CD4+CD25+ Treg-specific marker or a combination of markers such as FoxP3 and CD127, must be agreed upon and used to ensure the purity and correct identification of CD4+CD25+ Tregs.
2. It must be established that administration or manipulation of CD4+CD25+Tregs will only regulate specific, localized targets and not cause systemic immunosuppression. This may require the derivation of antigen-specific Tregs, which is possible only when an autoantigen is clearly defined.
3. When designing a therapeutic regimen it may be prudent to use CD4+CD25+ Tregs in combination with another treatment as administration or manipulation of CD4+CD25+ Tregs alone may not be enough to cure an established disease.
4. Administration of large numbers of CD4+CD25+ Tregs should be done with caution as high numbers of CD4+CD25+ Tregs could leave the recipient vulnerable to infections or tumours. Therapies which allow the in vivo expansion of CD4+CD25+ Tregs may be a better option. There are several promising new approaches for tolerance induction, which have been studied in humans, such as mucosal peptide-specific immunotherapy, T cell vaccination and tolerogenic dendritic cells. Genetic manipulation of T cells to generate Tregs is also a promising therapeutic tool.

Infectious diseases

A number of immunotherapeutic strategies to control HIV infection have revealed a possible antagonistic role for Tregs. This necessitates investigating ways to counteract the suppressive function, such as through Treg depletion or blockade of specific Treg immunosuppressive mechanisms, without further increasing the cellular immune activation associated with chronic HIV infection. Simply applying Treg immunotherapeutic strategies used in diseases other than HIV may pose problems caused by the complexity of HIV immunopathogenesis. There is evidence to support the hypothesis that Tregs induced by environmental mycobacteria do indeed constrain immunity induced by BCG. If verified in humans, the study opens up the possibility of developing improved TB vaccines based on manipulating immune responses by removing or suppressing Tregs during immunization.
Allergic diseases
Compelling evidence in the literature suggests that Tregs are ideal candidates for developing effective therapies to treat allergic diseases such as asthma.

Retinoic acid and vitamin D3 have been shown to directly enhance suppressive function of Tregs. Allergen-specific immunotherapy is yet another strategy to expand Treg repertoire in vivo. Currently, clinical data are available for two allergens that have been targeted with this approach—the cat allergen (Fel d1) and the bee venom allergen (Api m1) (phospholipase A2). A more conclusive study recently demonstrated that an inhaled corticosteroid could reverse the observed poor numbers of CD4+CD25high cells in bronchoalveolar lavage fluid of asthmatic children.

These agents induce short-term upregulation of FoxP3 expression and Tregs in patients with asthma. Rapamycin, an immunosuppressive drug, has been found to promote in vitro expansion of both murine and human Tregs, which could be important in generating clinically relevant quantities of Tregs.60

Infection by a number of microorganisms has been associated with reduced prevalence of asthma, and many infectious agents have been shown to induce Tregs and reduce allergic airway disease in mouse models. The translation of the regulatory and therapeutic properties of infectious agents for use in asthma requires the identification of key modulatory components and the development and trial of effective immunoregulatory therapies. Induction of Tregs by infectious agents and their components is thus another effective strategy to ameliorate allergic diseases. Although this would seem a straightforward approach, there are numerous issues that need to be addressed. The key components of infectious agents need to be identified and developed into immunoregulatory therapies, and administration regimens (dose, timing and route) would need to be optimized. Helminthic infections with Litomosoides sigmodontis, Nippostrongylus brasiliensis, Schistosoma japonicum and Schistosoma mansoni have been shown to induce Tregs and suppress allergic airways disease.67

CONCLUSION
Since the reappraisal of suppressor T cells by the pioneering work of Sakaguchi’s group, the field of immune control by Tregs has been progressing exponentially. Recent progress in the understanding of Treg biology, insights into the biological role of FoxP3 and the development of experimental mouse models have highlighted potential avenues in the translation of research-based knowledge to the clinic. However, despite recent advances, several major questions still remain. The quest for more specific markers on nTregs or iTregs will ultimately lead to improved methods to isolate and functionally characterize these subsets of Tregs. Better insights will then improve the design of new and better immunotherapies. This will open up avenues for exploration of novel and improved forms of treatment for various clinical conditions.

A number of questions regarding the origin, nature and mechanism of action of CD4+CD25+ Tregs are not clearly understood. As we improve our understanding of these questions and other issues related to Treg biology, we may understand how Treg function can be modulated in vivo. No single, uniquely expressed cell surface molecule has thus far been identified for Tregs. These could potentially help in in vivo and in vitro expansion for cellular therapy and functional studies. Elucidation of more specific phenotypic biomarkers of Tregs is currently the subject of intense research. There is also need to develop more standardized functional assays for Tregs.

There is a fine balance between benefit and harm of manipulating Tregs. Blocking Tregs functionally or numerically is a powerful strategy in cancer immunotherapy, but may also potentially induce autoimmunity due to cross-reaction between antigens expressed on tumours and normal tissue antigens. This obstacle needs to be circumvented. Moreover, obtaining remission from cancer by manipulating Tregs is in its infancy, but the early signs are encouraging. Maximum tolerable dose and safety profile of Treg-depleting agents needs to be evaluated. Preliminary results indicate the potential for combining Treg-depletion with antitumour cancer vaccines to enhance tumour antigen-specific immune response and the need to explore the dose and schedule of Treg-depletion strategies in optimizing vaccine efforts.

Reliable and sufficient protocols for in vitro expansion of human Tregs need to be established and their application in allogeneic transplantation needs to be explored further in clinical trials.

On the basis of data in murine models and limited human studies, modulating Tregs during chemotherapy, before vaccination or pre/post-transplantation or cellular therapy seems to be an attractive option that warrants further investigation in humans. However, due to the potential for severe adverse effects in the form of autoimmune disorders, vigilance for unexpected results will be required in designing clinical trials for Treg manipulation with novel agents.

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Conflict of interest. None declared

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