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Making Sense of Regulatory T Cell Suppressive Function

Itay Shalev¹, Moritz Schmelzle², Simon C. Robson^{2,*}, and Gary Levy^{1,*}

¹Multi-Organ Transplant Program, Toronto General Hospital, University Health Network, University of Toronto, Toronto, Ontario, Canada M5G 2N2

²Gastroenterology and Transplant Institute, Beth Israel Deaconess Medical Centre/Harvard Medical School, Boston, MA, 02215, USA

Abstract

Several types of regulatory T cells maintain self-tolerance and control excessive immune responses to foreign antigens. The major regulatory T subsets described over the past decade and novel function in transplantation will be covered in this review with a focus on CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells. Multiple mechanisms have been proposed to explain how Treg cells inhibit effector cells but none can completely explain the observed effects in toto. Proposed mechanisms to explain suppressive activity of Treg cells include the generation of inhibitory cytokines, induced death of effector cells by cytokine deprivation or cytotoxicity, local metabolic perturbation of target cells mediated by changes in extracellular nucleotide/nucleoside fluxes with alterations in intracellular signaling molecules such as cyclic AMP, and finally inhibition of dendritic cell functions. A better understanding of how Treg cells operate at the molecular level could result in novel and safer therapeutic approaches in transplantation and immune-mediated diseases.

Keywords

Regulatory T cells; Transplantation; Tolerance; Foxp3; CD39

Major roles in the maintenance of peripheral tolerance have been attributed to the activity of suppressor or regulatory T cells. Studies by Gershon et al. nearly 40 years ago first described the existence of suppressor T cells that could downregulate immune responses of antigen-specific T cells [1]. Around the same time, thymectomy studies suggested the generation of thymic-derived natural regulatory T cells that maintained immunological tolerance. In 1969, Nishizuka et al. noted that in mice, neonatal thymectomy induced an ovarian autoimmune disease [2]. Furthermore, Penhale et al. reported in 1973 that adult thymectomy of normal rats followed by sublethal X-irradiation also resulted in the development of autoimmune thyroiditis associated with tissue-specific autoantibodies [3]. Reconstitution of thymectomized animals with normal T cells inhibited autoimmunity [4].

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*Corresponding Authors: Simon C. Robson, Gastroenterology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue E/CLS; Room 0612, Harvard Medical School, Boston, Massachusetts 02215, srobson@bidmc.harvard.edu & Gary Levy, Multi-Organ Transplant Program, Toronto General Hospital, University Health Network, University of Toronto, Toronto, Ontario, Canada M5G 2N2, glfgl2@attglobal.net.

*Joint senior authors

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There were major issues at that time which precluded continued investigation of putative regulatory T cells. These included the lack of reliable and validated markers for the identification of these cells together with great difficulty in isolating cell clones and defining peripheral cell effects [5]. The initial enthusiastic and intensive investigations of suppressor T cells therefore declined in the late 1980s.

However, in 1995 a seminal paper published by Sakaguchi et al reinvigorated the field [6]. In recent years with the development of advanced molecular and cellular tools, several new cell markers were identified and suppressive functions of multiple cytokines were characterized resulting in the reappraisal of regulatory T cells as mediators of self-tolerance [5].

The existence of various regulatory T cell subsets has been demonstrated, which express distinct cytokines or receptors and function by diverse pathways at differing stages of the immune response [7]. The proposed suppressive functions of regulatory T cells include: generation of inhibitory cytokines; death of effector cells by cytokine deprivation or cytotoxicity; local metabolic perturbation of target cells mediated by changes in extracellular nucleotide/nucleoside fluxes with alterations in intracellular signaling molecules such as cyclic AMP, and finally inhibition of dendritic cell functions [8].

It is generally accepted that regulatory T cells comprise two major subsets based on presumed ontogeny: adaptive regulatory T cells, which are induced in the periphery in response to antigen stimulation under tolerogenic conditions, and naturally occurring regulatory T cells, which are constantly produced by the thymus. Together these regulatory T cells maintain self-tolerance and control excessive immune responses to foreign antigens in the periphery. The major regulatory T cell subsets described over the past decade will be reviewed here (Figure 1); with a focus on the most extensively studied regulatory T cell subset viz. CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells.

Regulatory T cells type 1 (Tr1)

Tr1 cells are unusual as these regulatory T cells do not express high levels of either CD25 or Foxp3. These cells were first described in patients with severe combined immunodeficiency (SCID), who had been successfully transplanted with allogeneic hematopoietic stem cells (HSCs). CD4⁺ host-reactive T cell clones that were isolated from these SCID patients produced high levels of IL-10 and low amounts of IL-2 after antigen stimulation *in vitro*. Immunologic reconstitution and induction of tolerance following HLA-mismatched transplantation of hematopoietic stem cells (HSC) were associated with the presence of IL-10-producing CD4⁺ T cells [9]. Subsequent studies by Groux et al. demonstrated that *ex vivo* activation of human and murine CD4⁺ T cells in the presence of high levels of IL-10 induced the generation of IL-10-producing CD4⁺ T cells with low proliferative responses. These cells produced a unique set of cytokines, characterized by high levels of IL-10, TGF- β and IL-5 but low levels of IL-2 and IFN- γ , and no IL-4 [10]. Due to their ability to suppress T-cell immune responses *in vitro* and *in vivo*, IL-10-producing CD4⁺ T cells were termed regulatory T cells type 1 (Tr1) and had the recognized potential to prevent colitis (8).

Tr1 arise in the periphery following activation of naïve T cells with antigen in the presence of IL-10, and act as important regulators of adaptive immune responses through the suppression of naïve and memory T cell-responses with associated inhibition of antigen presenting cell or dendritic cell (DC) stimulatory activities [11]. Although Tr1 are induced in response to antigenic-specific stimulation, they exert their suppressive function in an antigen non-specific manner through the production of IL-10 and TGF- β . The release of the anti-inflammatory cytokines IL-10 and TGF- β is likely the reason for the bystander suppression of Tr1 cells [11].

The regulatory activity of Tr1 cells has been implicated in various immunopathologies both in mice and humans, inclusive of intestinal inflammation [11]. The importance of Tr1 cells in the maintenance of self-tolerance has been demonstrated. Isolated Tr1 cells bearing self-MHC reactive TCR from the peripheral blood of apparently healthy individuals have been shown to suppress proliferative responses of autoreactive T cells in an antigen-specific manner via production of IL-10 and TGF- β [12]. Furthermore, the numbers of Tr1 cells isolated from peripheral blood and synovial tissues of patients with rheumatoid arthritis are significantly lower compared to control patients [13]. The presence of Tr1 cells also correlates with the absence of graft-versus-host-disease (GVHD) and long-term graft tolerance in SCID patients transplanted with allogeneic HSCs [14]. In addition, a high frequency of Tr1 cells was associated with the absence of acute GVHD following bone-marrow transplantation (BMT), while low proportions correlate with development of severe GVHD [15]. Moreover, acceptance of kidney and liver allografts has been associated with the presence of Tr1 cells, which suppress naive T cell responses through the production of IL-10 and TGF- β [16].

Clinical trials are currently ongoing to evaluate the potential therapeutic effects of Tr1 cells in the prevention and treatment of GVHD post BMT [17]. The clinical protocol involves the transfer of ex vivo-generated Tr1 cells to patients with hematological cancers treated with HSC transplantation. Treatment of the host with the IL-10-anergized donor T cells has potential to reconstitute the immunity without GVHD while guarding against infection and recurrence of cancer [17]. Success in such clinical trials might pave the way for Tr1-based immunotherapy in other immune-mediated diseases, such as autoimmunity and allergy.

T helper 3 (Th3)

Early studies of oral tolerance led to the identification of a unique CD4⁺ T cell subset, which was later referred to as T helper type 3 (Th3) cells which express TGF- β . Such Th3 cell subpopulations were induced in the gut-associated lymphoid tissues of SJL mice by oral administration of myelin basic protein (MBP). These cells were able to suppress the proliferation and cytokine production of MBP-specific Th1 cells, and inhibit the development of experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis [18]. Production of TGF- β by Th3 cells was shown to account for the inhibition of EAE, as antibodies to TGF- β blocked the suppressive activity of Th3 cells [18]. In subsequent studies, Th3 cells were also identified in multiple sclerosis patients who were orally treated with MBP and proteolipid protein (PLP), which increased the frequency of TGF- β -secreting Th3 cells specific for MBP and PLP [19].

The gut microenvironment with high levels of TGF- β and Th2 cytokines, as well as unique subsets of DC, promotes the development of Th3 cells upon encounter with oral antigens [20]. Generation of Th3 cells is thought to be important in the induction and maintenance of tolerance to non-pathogenic resident bacteria and potentially immunogenic food antigens. Th3 cells are activated in response to specific antigens but suppress in an antigen-nonspecific manner through the release of TGF- β , and therefore mediate bystander suppression. Th3-type cells down regulate both Th1 and Th2 cells and provide help for B cells in the production of IgA antibodies [20].

The regulatory activity of Th3 cells has been implicated in the control of various experimental autoimmune disease models other than EAE, including spontaneous autoimmune diabetes, experimental autoimmune myasthenia gravis and autoimmune glomerulonephritis [21]. The importance of Th3 cells was also reported in donor transfusion-induced allograft tolerance and in suppression of lung eosinophilic inflammation [20]. Recent studies have also demonstrated a role for Th3 cells in some cases of human

autoimmunity and allergy, as reduction in the number of the TGF- β -producing T cells or production of TGF- β was found in the duodenal mucosa of children with food allergy and patients with active chronic idiopathic thrombocytopenic purpura, respectively [22;23].

CD8⁺ regulatory T cells

In recent years several types of CD8⁺ T cells with regulatory or suppressive function have been identified. These include antigen nonspecific cells that are naturally produced in the thymus and antigen specific cells that are generated in response to foreign or self-antigens during the course of the peripheral immune response. In addition, other antigen specific and nonspecific subsets of CD8⁺ regulatory T cells have also been induced *in vitro* [24].

Recent studies defined CD8⁺CD25⁺ human thymocytes that share similar phenotypic and functional properties with naturally occurring CD4⁺CD25⁺ T cells produced by the thymus [25]. CD8⁺CD25⁺ thymocytes express increased mRNA levels of Foxp3, glucocorticoid-induced tumor necrosis factor receptor (GITR), CCR8, TNFR2 and CTLA-4. Following activation, these cells do not produce cytokines but express surface TGF- β 1 and CTLA-4. Purified CD8⁺CD25⁺ thymocytes were found to be anergic *in vitro*, and were capable of suppressing the proliferation of CD4⁺ effector T cells in a contact-dependent manner. Antibodies to TGF- β 1 and CTLA-4 abrogate functions of these regulatory cells [25]. A similar population of CD8⁺CD25⁺ T cells was also detected in the periphery and thymus of mice lacking MHCII. This cell population expressed Foxp3, CTLA-4 and IL-10, and was also shown to regulate the response of both CD4⁺ and CD8⁺ effector T cells to anti-CD3 stimulation [26].

In additional studies, autoreactive human peripheral blood CD8⁺CD25⁺Foxp3⁺ T cells have been expanded and cloned following the stimulation with autologous LPS-activated DC. These cells proliferate and produce anti-inflammatory cytokines, including TGF- β 1 and IL-4, upon stimulation with DC in an autoreactive HLA-restricted manner. A CD8⁺CD25⁺Foxp3⁺ clone inhibited proliferation and IFN- γ production of CD4⁺ T cells *in vitro* through CTLA-4 in a cell-to-cell contact [27].

A distinct population of human CD8⁺ Foxp⁺ regulatory T cells can be induced following repeated stimulation of peripheral blood mononuclear cells *in vitro* with allogeneic, xenogeneic or self-APCs pulsed with antigen [28;29]. These cells exert regulatory activity by interacting directly with DC, monocytes and endothelial cells (ECs). This interaction induces the upregulation of the inhibitory receptors, immunoglobulinlike transcript 3 (ILT3) and ILT4 on DC, leading to inhibition of NF- κ B activation and decreased expression of costimulatory molecules CD80 and CD86. The suppressed APCs become tolerogenic and are therefore incapable of inducing and sustaining the full activation of T helper cells [30].

Previous studies have suggested a role for CD8⁺CD28⁻ T cells in the inhibition of allograft rejection both in animals and humans [28;31] and in suppression of EAE [32]. A novel subset of natural regulatory CD8⁺ T cells has been also described in normal healthy animals. These CD8⁺ T cells express low levels of surface CD45RC and following stimulation *in vitro* generate predominately T helper type 2 cytokines, including IL-4, IL-10 and IL-13 [33]. These cells also express the transcription factor Foxp3 and CTLA-4. CD8⁺CD45RC^{low} T cells inhibit the proliferation and differentiation of CD4 cells into Th1 cells in response to allogeneic DC via a cell-to-cell contact. These regulatory cells protect against the development of GVHD induced by CD4⁺ T effector cells in rats [33]. A recent report also showed that allograft acceptance in major MHC-mismatched cardiac allograft model in rats is mediated by the regulatory activity of CD8⁺CD45RC^{low} T cells [34]. It was proposed that CD8⁺CD45RC^{low} T cells act through the secretion of IFN- γ that in turn induces production of indoleamine 2,3-dioxygenase (IDO) by graft ECs. The immunoregulatory enzyme IDO

catalyzes the essential amino acid tryptophan required for the growth of T cells, and expression therefore suppresses alloreactive T cell responses and promotes allograft tolerance [34].

CD8⁺CD122⁺ T cells are defined as naturally occurring IL-10-producing regulatory T cells [35–37]. They directly suppress the proliferation and IFN- γ production of both CD4⁺ and CD8⁺ T cells *in vitro*. CD8⁺CD122⁺ T cells also have important regulatory function *in vivo* as indicated by their ability to suppress EAE and prevent the development of abnormal T cells in CD122-deficient mice [36;38]. This subset exerts their function mainly through the production of IL-10. Deletion of the *IL-10* gene or antibody against IL-10 abrogates the suppressive activity of CD8⁺CD122⁺ T cells *in vitro*. However, *IL-10*^{-/-} CD8⁺CD122⁺ T cells are found to have some regulatory activity *in vivo*, suggesting that additional factors may contribute to the function of these cells [35].

A second class of IL-10-producing inducible CD8⁺ regulatory T cells have been reported *in vitro* [39]. Gilliet et al. showed that stimulation of naive CD8⁺ T cells with allogeneic CD40 ligand-activated plasmacytoid DC (DC2) resulted in the generation of these regulatory T cells. These cells produced high levels of IL-10 and low IFN- γ , and generation was dependent on the presence of IL-10 in the cell culture. DC2-primed Treg cells were able to suppress allospecific proliferation of naive CD8⁺ T cells in response to monocytes and DC. Similar to CD8⁺CD122⁺ T cells, the suppressive activity of DC2-primed CD8⁺ T cells can be mediated, at least in part, by the production of IL-10 [39].

Another distinct regulatory T cell subset expressing TCR $\alpha\beta$ and CD8 $\alpha\alpha$ has been recently defined [40;41]. High frequencies of these cells have been detected in the intestinal intraepithelial lymphocyte (IEL) population of the gut (40%), and low numbers are also found in the spleen and lymph nodes (<1%). Previous studies showed that TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ Treg cells are derived from the thymus by exposure to self-agonists, and that these cells are self-reactive. Abundant transcript levels of TGF- β 3, lymphocyte activation gene-3 (LAG-3) and fibrinogen-like protein 2 (FGL2) have been detected in TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ Treg cells of the IEL population [42]. Due to the increased expression of genes that are involved in immune regulation and the self-reactivity of IEL CD8 $\alpha\alpha$ ⁺ Treg cells, it was proposed that these lymphocytes might play a role in regulation of mucosal immunity [42].

In support of this concept, Poussier et al. demonstrated that TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ Treg cells prevented colitis induced by CD4⁺CD45RB^{high} T cells in SCID mice. This inhibitory activity was dependent on the production of IL-10, as TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ Treg cells from IL-10-deficient mice could not efficiently prevent the disease [43]. In additional experiments, it was shown that TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ Treg cells can inhibit the development of EAE [40]. Further studies are required to evaluate the relationship between these subsets and determine whether these cells represent distinct regulatory cells or are representative of cells under different conditions of activation.

Double negative T cells

In rodents, CD4⁻CD8⁻CD3⁺ regulatory T (DN Treg) cells constitute 1–3% of peripheral T cells [44]. These DN Treg cells express a unique set of cell surface markers, including TCR $\alpha\beta$, CD25, LFA-1, CD69, CD45, CD30, CD62L and CTLA-4. Activated DN Treg cells also exhibit a distinct cytokine profile, characterized by the production of increased amount of IFN- γ , TNF- α and low levels of TGF- β . However, production of IL-2, IL-4, IL-13 or IL-10 has not been detected in these cells [45].

DN Treg cells can suppress both CD4⁺ and CD8⁺ T cell-mediated immune responses *in vitro* and *in vivo*. The regulatory activity of DN Treg cells has been implicated in a number

of experimental transplant and autoimmune disease models. Previous studies showed that stimulation of DN Treg cells with donor antigens results in inhibition of anti-donor T cell responses and enhance allograft and xenograft survival in an antigen-specific manner [46;47]. In additional experiments, it was also demonstrated that treatment with activated antigen-specific DN Treg cells can prevent the development of GVHD and autoimmune type 1 diabetes induced by pathogenic CD8⁺ T cells in a mouse model [48;49].

The mechanism by which DN Treg cells exert their regulatory activity has been extensively studied. DN Treg cells act directly on effector T cells in an antigen-specific manner through a process that requires cell-to-cell contact. Prior to encountering target cells, DN Treg cells acquire via their antigen-specific TCR allo-MHC peptides from antigen presenting cells. Presentation of the acquired alloantigens on DN Treg cells enables them to induce apoptosis of CD4⁺ and CD8⁺ T cells that express the same TCR specificity as DN Treg cells. Killing of effector T cells by DN Treg cells is mediated predominately through Fas/Fas-ligand interactions, although other additional pathways may contribute to the regulatory activity of this population [50].

The origin and development of DN Treg cells have not been well defined; however, recent reports have shed light on the underlying mechanisms that are involved in the generation of DN Treg cells. Ford et al. reported that peripheral DN Treg cells can be found in the spleen and lymph nodes of thymectomized mice, irradiated and reconstituted with T cell-depleted bone-marrow (BM) cells. These studies suggest that DN Treg cells can develop outside of the thymus [51]. Indeed, Zhang et al. have identified a new differentiation pathway for the conversion of CD4⁺ T cells to DN Treg cells [52]. Peripheral CD4⁺ T cells can downregulate CD4 expression and become DN Treg cells via auto/alloantigen-triggered or homeostatic proliferation. Differentiation of DN Treg cells from mature CD8⁺ T cell population was not observed [52]. These findings reveal a novel intrinsic homeostatic pathway that provides a deeper understanding of the peripheral regulatory mechanisms of the immune system.

DN Treg cells have also been identified and characterized in humans [53]. These cells comprised 1–2% of total CD3⁺ T cells in the blood and lymph nodes of healthy individuals. Similar to DN Treg cells in mice, activated human DN Treg cells generate high levels of IFN- γ , but no IL-2 and very low levels of IL-10 and IL-4. Human DN Treg cells have been shown to exhibit similar functional properties as murine DN Treg cells. DN Treg cells were able to acquire MHC-peptide complexes from APCs and use these to induce apoptosis and suppress proliferation of antigen-specific cytotoxic T lymphocytes (CTL) [53]. It was recently reported that human DN Treg cells may also play a role in preventing GVHD in patients after allogeneic HSC transplantation [54]. Taken together, the results obtained from human and animal studies may potentially lead to the development of novel cell-based therapies using DN Treg cells to prevent allograft rejection and treat autoimmune diseases.

$\gamma\delta$ regulatory T cells

Approximately, 5% of total T cells in the peripheral lymphoid tissues express TCR gamma/delta, which is composed of γ and δ chains instead of the conventional $\alpha\beta$ heterodimers. TCR $\gamma\delta$ ⁺ T cells are enriched in the intraepithelial lymphocyte (IEL) compartments of skin, intestine and genitourinary tract, where these cells represent up to 50% of total T cells [55]. $\gamma\delta$ T cells may be considered part of the adaptive immune response since these cells rearrange TCR genes and develop into memory cells. The $\gamma\delta$ T cells may also be considered a component of the innate immune system, as they display restricted TCR repertoires that can respond to pathogen associated molecular patterns (PAMP) and self-antigens. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells are not MHC-restricted; their development can be thymic

dependent or independent and these cells are further capable of recognizing soluble protein and non-protein antigens of endogenous origin [55].

$\gamma\delta$ T cell function has been implicated in downregulation of immune responses associated with pathogenic infection, allergens and autoimmune diseases [56]. Mice that lack $\gamma\delta$ T cells exhibit exaggerated or accelerated immunopathology when infected with intestinal *Eimeria*, *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Klebsiella*. In addition, $\gamma\delta$ T-cell-deficient mice on a specific background can develop a spontaneous $\alpha\beta$ T cell-dependent cutaneous inflammation (dermatitis), as well as augmented responses to contact allergens and irritants [56]. The regulatory activity of $\gamma\delta$ T cells has also been reported in graft-versus-host disease (GVHD) and several experimental models of autoimmune disease, such as type 1 diabetes and lupus erythematosus [55]. It is important to note that in certain infections and immunopathologies, $\gamma\delta$ T cells may also display a pro-inflammatory phenotype. Therefore, it has been proposed that $\gamma\delta$ T cells could exert different functions depending on their tissue distribution, antigen receptor structure and local microenvironment [55].

A number of possible mechanisms whereby $\gamma\delta$ T cells mediate their regulatory activity have been proposed [57]. Production of chemokines and anti-inflammatory cytokines by $\gamma\delta$ T cells (such as TGF- β , IL-10 and thymosin- β 4) can regulate the local homing and maturation of other lymphoid cells. Another possibility is that $\gamma\delta$ T cells through the release of IFN- γ induce upregulation of Fas ligand receptors on epithelia that then targets pathogenic immune cells infiltrating the tissue. The $\gamma\delta$ T cells may also directly kill effector lymphoid cells through Fas-Fas ligand interaction. Finally, it was suggested that secretion of epithelial growth factors (keratinocyte growth factor) by $\gamma\delta$ T cells may contribute to epithelial tissue repair, which in turn provides a physical and chemical barrier to systemic infiltration of activated immune cells [57].

Natural killer T cells

Natural killer T (NKT) cells co express NK-cell-surface receptors (NK1.1/CD161) with the semi-invariant T-cell receptors (TCR). This TCR consists of an invariant α chain preferentially paired with restricted variable β chains [58]. In mice, most NKT cells have an invariant V α 14-J α 18 rearrangement paired with V β 8.2, V β 2 or V β 7 chains, while the homologous population of human NKT cells expresses an invariant V α 24-J α 18 rearrangement paired with V β 11. In contrast to other T lymphocytes, which recognize peptides in the context of classical MHC class I or II molecules, NKT cells interact with glycolipids presented by the non-classical MHC class I-like molecule CD1d [59]. Upon activation, NKT cells produce large amount of Th1 (including IFN- γ and TNF- α) and Th2 (IL-4, IL-10 and IL-13) cytokines enabling them to act as powerful regulators of the immune system.

In addition to their role in tumor rejection and resistance to pathogenic infection, NKT cells have also been implicated in promotion of tolerance induction in various autoimmune disorders and in allograft acceptance [60]. Data obtained from animal models of multiple sclerosis (MS) and type-1 diabetes have provided the best evidence for the role of NKT cells in inhibition of autoimmune diseases. The protective effect of NKT cells in these well-characterized models is typically associated with either Th2 polarization or a suppression of autoreactive T cells. Experimental mouse models also showed that NKT cells were required for allograft tolerance and inhibition of GVHD, involving NKT cell-dependent generation of downstream allospecific Treg cells and IL-4 production, respectively [60].

The relevance of NKT cells in autoimmune diseases has been reported by a number of studies. Reduced numbers of V α 24 NKT cells have been associated with the development of type-1 diabetes, MS, systemic lupus erythematosus, systemic sclerosis and rheumatoid

arthritis [61]. Similar to the murine studies, it appears that human NKT cells mediate their suppressive activity by shifting immune responses from Th1 to Th2 immunity.

However, in certain experimental conditions, it has been shown that NKT cells can contribute to the pathogenesis of diseases. For example, Concanavalin A induced hepatic injury has been shown to be NKT cell-mediated, as used in studies of immune-mediated liver disease. In a recent report [62], we have described expression of ectonucleotidases of the CD39 and CD73 family as well as membrane nucleotide or purinergic type-2 receptors on hepatic NKT cells. We have noted that in a manner analogous to Foxp3⁺ CD4⁺ Treg cells (see later) that NKT cells generate extracellular adenosine that might be expected to exert immune suppressive effects on target cells expressing adenosine A2A type-1 receptors. Heightened levels of apoptosis of CD39 null NKT cells are noted *in vivo* and *in vitro* which appear to be driven by unimpeded activation of the P2X7 receptor by extracellular ATP. Hence, *in vivo*, mice null for CD39, the major ectonucleotidase on NKT cells, are paradoxically protected from immune liver injury. CD39 null mice show substantively lower serum levels of transaminases, IL-4 and IFN- γ , when compared with matched wild-type mice administered Concanavalin A or aGalCer.

Clearly, further studies are required to evaluate the factors that determine the net outcome of NKT cell function in different diseases. A better understanding of the molecular mechanisms whereby NKT cells exert their immunoregulatory activities may help for the development of novel potential therapies for the treatment of a wide range of diseases.

CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells

Naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells were (re)discovered over a decade ago by studies of Sakaguchi et al. [6] and their existence confirmed by others (refs 63, 65 etc). Sakaguchi et al. noted that a small but distinct CD4⁺ T cell subset (5–10% of peripheral CD4⁺ T cells and 5% of CD4⁺CD8[−] thymocytes), which expresses high levels of the IL-2 receptor α -chain (CD25) and has suppressive properties. Athymic nude mice that were infused with syngeneic splenic T cells depleted of these Treg cells developed autoimmune diseases including thyroiditis, gastritis, insulinitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis and polyarthritis. Co-transfer of a small number of these putative Treg cells with CD25[−] splenic T cells prevented the development of autoimmune disease, in a dose-dependent manner [6]. Infusion of Treg cells was also shown to inhibit excessive immune responses of effector T cells to allo and xeno antigens in nude mice. Moreover, the appearance of Treg cells in the periphery of normal mice 3 days after birth correlated with the development of autoimmunity in mice that were thymectomized around the same day. Reconstitution of these mice with Treg cells inhibited the autoimmune disease. Taken together, these early studies demonstrated for the first time the existence of thymic-derived Treg cells that are essential for the maintenance of self-tolerance and negative control of immune responses to non-self antigens.

Shortly after the discovery of CD25 as a marker for Treg cells, *in vitro* assays were established to study immune functions of Treg cells [63]. Using these *in vitro* assays it was shown that Treg cells can suppress the proliferation of stimulated CD4⁺ and CD8⁺ T cells in the presence of DC. Treg cells were found to be anergic as these cells failed to produce IL-2 upon stimulation. However, TCR-stimulated Treg cells were capable of proliferating in the presence of high levels of IL-2. Further *in vitro* assays also showed that Treg cells act through a direct contact with target cells, without the need of soluble factors. The simple and highly reproducible *in vitro* assay together with CD25 discovery eventually led to the identification of human CD4⁺CD25⁺ Treg cells with phenotypic properties comparable to those found in rodents [64].

Expression of CD25 and the presence of IL-2 are critical for the generation and maintenance of Treg cells. Accordingly, deletion of CD25 or IL-2 in mice and genetic deficiency of CD25 in humans result in decreased numbers of Tregs and development of severe autoimmune disorders. Furthermore, treatment of naive normal mice with neutralizing antibody to circulating IL-2 causes decreases in Treg cell numbers and provokes autoimmunity [65]. The main source of IL-2 responsible for Treg cell survival and activation *in vivo* is derived from activated effector T cells, and it was therefore proposed that IL-2 production in sites of inflammation serves as a negative feedback that regulates and controls adaptive immune responses [65].

Role of Foxp3 as a master regulator of Treg cells

Rudensky, Sakaguchi and colleagues have identified the specific expression of Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors, in CD4⁺CD25⁺ Treg cells [66;67]. This transcription factor has been described as a master switch or regulator for Treg cell development and function. Disruption of *Foxp3* leads to impaired generation and activity of Treg cells and results in a fatal lymphoproliferative disorder in mice. Similarly, humans with mutations in the gene encoding Foxp3 suffer from a genetic disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is characterized by multiorgan autoimmune disease, allergy and a variant form of inflammatory bowel disease (IBD) [68].

The importance of Foxp3 as a key control molecule in Treg cells was further supported by recent studies, which demonstrated the ability of Foxp3 transduction to induce a Treg cell phenotype in naive T cells with a characteristic molecular profile or signature [69]. Foxp3-transduced cells exhibited regulatory activity, hyporesponsiveness upon stimulation, low production of IL-2 and upregulation of Treg-associated molecules (such as CD25, CTLA-4 and GITR) [69]. Several reports have recently suggested that Foxp3 controls Treg lineage development through direct or indirect activation and repression of hundreds of genes. Foxp3 appears to regulate gene expression by forming a transcription complex with other key transcription factors, such as NFAT, NFκB, AP-1 and AML/Runx1. The target genes include those that encode nuclear factors controlling gene expression and chromatin remodeling, plasma membrane proteins and ectoenzymes that hydrolyze extracellular nucleotides, as well as cell signaling proteins [70].

In addition to natural Foxp3⁺ Treg cells that are constantly produced by the thymus, naive T cells in the periphery may also acquire Foxp3 expression and convert into Treg cells with functional and phenotypic similarity to natural Treg cells counterpart. Induced Treg (iTreg) cells may arise following *in vitro* antigenic stimulation of naive T cells in the presence of TGF-β. Both IL-2 and retinoic acid (a vitamin A metabolite) promote TGF-β-dependent generation of iTreg, while IL-6 inhibits this process and induces differentiation of naive CD4⁺ T cells into Th17 inflammatory cells [71]. Chronic antigenic stimulation could also induce Foxp3⁺ Treg cells in the periphery through a process that involves antigen presentation in the absence of a 'danger signal', such as may occur with food antigens in the gut [24]. Further studies are still required to assess the long-term stability of iTreg in the periphery, and to evaluate their contribution to the peripheral pool of Foxp3⁺ Treg cells and their physiological significance in the induction of tolerance [71].

The identification of Foxp3 as a specific marker for Treg cells has revealed two major differences between human and mouse Treg cells. In humans, naive T cells can readily express low and transient levels of Foxp3 upon TCR stimulation, whereas similar patterns of expression in mouse Treg cells have not been observed. The induced expression of Foxp3 in human naive T cells, however, does not confer suppressive function, and may serve as a T-

cell-intrinsic mechanism that can regulate the development of pathogenic T cells [72]. Furthermore, expression levels of CD25 and Foxp3 correlate well in murine Treg cells (90% of CD25⁺ T cells are Foxp3 positive) allowing for their efficient isolation based on the expression of high levels of CD25. In contrast, human Foxp3⁺ Treg cells can either express high or intermediate levels of CD25 which make their isolation more difficult, given the fact that CD25 at various levels can also be detected on activated T cells [24]. Collectively, these data suggest that the analysis and isolation of human Treg cells based on Foxp3 and CD25 levels should be performed with caution.

Functions of Treg cells

With the discovery of Foxp3 and CD25 allowing for direct molecular and functional investigation of Treg cells, there is now compelling evidence in mice and humans that these cells are critical for the negative control of various pathological and physiological immune responses [73]. In recent years many laboratories worldwide have demonstrated the importance of Treg cells in the prevention of a broad spectrum of autoimmune diseases (in experimental models for type 1 diabetes, MS, rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus), as well as the inhibition of GVHD and allograft rejection. Treg cells have also been implicated in the negative control of excessive immune responses associated with pathogenic infection and cancer, which in some cases could be harmful to the host leading to chronic infection and cancer progression [73].

Manipulation of Treg cells in the clinical settings provides a novel therapeutic approach for the treatment and prevention of a variety of human diseases. For example, increasing Treg cell number or enhancing their suppressive activity may lead to the inhibition of autoimmunity and the induction of tolerance to non-self antigens, including allografts, allergens and commensal bacteria. On the other hand, a reduction in the number or function of Treg cells could augment host immunity against bacterial infection and tumor cells, resulting in viral and tumor eradication (Figure 2) [71].

Clinical trials currently examine the ability of Treg cells to prevent GVHD following HSC transplantation [17]. In these trials, either freshly isolated Treg cells or ex-vivo expanded Treg cells (with CD3- and CD28-specific antibodies and high doses of IL-2) from the donor are infused into the transplant recipient to inhibit and prevent the development of the T cell-mediated pathology [17]. Recent studies by Ianni et al. in 28 patients with high risk hematological malignancies have shown that adoptive transfer of Treg cells prevented GVHD in the absence of any post-transplant immunosuppressant therapies [74].

Mechanisms of CD4⁺CD25⁺Foxp3⁺ Treg-mediated suppression

Treg cells possess TCRs that can recognize both self and non-self antigens, and appear in an activated state, as evident by the expression of high levels of activation markers and adhesion molecules [75]. Unlike the hyporesponsiveness exhibited *in vitro*, a large number of Treg cells continuously proliferate *in vivo* probably due to their interaction with self-antigens and commensal bacteria in the periphery. Antigenic stimulation is required to activate the suppressive function of Treg cells, however, once activated they down-regulate immune responses in an antigen-nonspecific manner [75]. The regulatory cells can suppress the proliferation and activation of many different types of immune cells, including T cells, DC, B cells, NK cells and NKT cells both *in vitro* and *in vivo* [71]. In the past few years there has been a significant progress in defining the effector molecules that Treg cells use to mediate their regulatory activity (Table 1).

Based on their molecular characterization and function, effector molecules of Treg cells can be classified into four groups: immunosuppressive cytokines, molecules involved in

metabolic signaling, cytolytic molecules and membrane-associated molecules that down-modulate the activation of effector cells (Table 2).

Immunosuppressive interactions via cytokines and receptors

Secretion of inhibitory cytokines has been proposed as a major pathway by which Treg cells exert their function. Among these inhibitory molecules, TGF- β and IL-10 have been reported as key mediators of Treg cell suppression [76]. Interestingly, TGF- β and IL-10 are both dispensable for the function of Treg cells *in vitro*, as their deletion or neutralization using a specific antibody does not alter Treg cell suppression [76]. However, the inhibitory role of TGF- β and IL-10 *in vivo* has been demonstrated in various experimental models, including IBD, Leishmania skin infection, type-1 diabetes, transplantation, and tumor models. Furthermore, loss of TGF- β in mice leads to the development of a lethal autoimmune disease associated with a reduction in Treg cell numbers, while mice that lack IL-10 display severe intestinal inflammation in response to normal flora [76;77]. The inhibitory cytokine IL-35, a member of the IL-12 family, has recently been described as a potent suppressive molecule of Treg cells. IL-35 is preferentially produced by Treg cells and its transcript levels are markedly up-regulated in Treg cells that are actively suppressing. The important contribution of IL-35 to Treg cell function has been demonstrated both *in vitro* and *in vivo* in an animal model of IBD [78].

In addition to the role of CD25 in the maintenance and generation of Treg cells, it has been suggested that high level expression of the receptor is used by Treg cells to absorb the local IL-2 required for non-Treg cell survival. The deprivation of the essential growth factor IL-2 leads to apoptosis of naive and effector T cells [79].

Fibrinogen-like protein 2 (FGL2) has been recently described as another potent suppressive molecule of Treg cells [80–82]. Expression of FGL2 by Treg cells leads to inhibition of DC maturation and T cell activation, and B cell apoptosis through binding to the inhibitory Fc γ RIIB receptor expressed on antigen- presenting cells [81]. Targeted deletion of *fgl2* in mice leads to increased reactivity of DC, T and B cell responses and the development of autoimmune kidney disease [83]. The immunoregulatory activity of FGL2 plays an important role in the pathogenesis of experimental and human viral hepatitis models [82;84;85]. In transplantation, treatment with recombinant mouse FGL2 results in prolongation of graft survival in a fully MHC-mismatched allotransplant murine model [81]. *Fgl2*-transgenic mice that ubiquitously over-express high levels of FGL2 accept fully mismatched heart allografts (unpublished data). In a heart transplant model of tolerance, FGL2 act as a key mediator of CD8⁺ Treg cell suppression [80].

Chemometabolic regulation

Several Treg effector molecules are involved in the homeostatic disruption of the effector cell-targets and directly perturb metabolic pathways and signaling. These molecules include the inhibitory second messenger cyclic AMP (cAMP), which can be transferred directly by Treg cells into target cells through membrane gap junctions. Upon entrance to the target cell, cAMP inhibits IL-2 production and proliferation [86].

Treg cells also indirectly boost cAMP levels in target cells by augmenting adenosine-mediated signaling via augmented generation of this nucleoside from extracellular nucleotides. We have recently explored the patterns of CD39/ecto-nucleoside triphosphate diphosphohydrolase expression and the functional role of this ectonucleotidase within quiescent and activated T cell subsets. Our data indicate that CD39, together with CD73 which is an ecto-5'-nucleotidase, more efficiently distinguishes Treg cells from other resting or activated T cells in mice (and humans) than do other suggested markers. Furthermore,

CD39 serves as an integral component of the suppressive machinery of Treg cells, acting, at least in part, through the modulation of pericellular levels of adenosine. These ectoenzymes catalyze the generation of pericellular adenosine that delivers a negative signal to effector cells by interaction with the adenosine A2A receptor [87]. The coordinated regulation of CD39/CD73 expression and of the adenosine receptor A2A also activates an immunoinhibitory loop that differentially regulates Th1 and Th2 responses. The *in vivo* relevance of this network is manifest in the phenotype of CD39-null mice that spontaneously develop features of autoimmune diseases associated with Th1 immune deviation [87;88] and exhibit severe experimental colitis [89]. This latter phenotype is also mirrored in patients with Crohn's disease who exhibit genetic polymorphisms that predispose to inflammatory bowel disease [89].

High levels of extracellular ATP after tissue injury are known to induce apoptosis via purinergic receptor P2X7 activation. Functionally active CD39 and CD73 have been demonstrated to protect Treg cells from P2X7-mediated apoptosis by regulated phosphohydrolysis of extracellular nucleotides [90]. In a manner similar to ATP, nicotinamide adenine dinucleotide (NAD) is also released into the extracellular environment by cell lysis and non-lytic mechanisms [91]. It seems that NAD also modulates survival, phenotype, and function of Treg cells [92]. NAD activates the P2X7 receptor by ART2.2-mediated ADP-ribosylation. NAD-dependent P2X7 receptor activation which ultimately results in T cell death, a process, at least in part, modulated by CD38 (NAD-glycohydrolase)-dependent hydrolysis of extracellular NAD (ADP-ribosylation of membrane proteins [93].

Recent studies in mice and humans suggest a complex role of CD39 in modulating T and NK(T) cell-mediated immune responses [62;88;94]. Interestingly, CD39 is also expressed by Foxp3^{low}CD4⁺ T cell populations that generate Th1, Th2 and Th17 cytokines [90]. In contrast to Treg cells (in the mouse), this memory population has been characterized by low levels of expression of CD73.

In humans, subsets of CD4⁺CD25⁺Foxp3⁺CD127^{low} Treg cells have recently been shown to be defined by robust expression of CD39, independently of CD73 [95]. Recent data in humans also suggest that CD39 expressing Treg cells suppress Th17 cells or preclude their generation and accordingly IL-17 production in disease states e.g. in multiple sclerosis and during renal transplant rejection [95;96].

Yan et al. [97] were the first to suggest that Treg cells may affect the extracellular redox state to suppress effector cells and recent work by others has just established that NADPH oxidase derived reactive oxygen species play a role in cell mediated immune suppression [98].

Treg cells are anergic and have been recently shown to express low levels of the glucose transporter Glut1 and to have high rates of lipid oxidation in contrast to effector cells that are highly glycolytic [99]. It is now thought that activated T cells switch to anabolic metabolism, characterized by fermenting glucose to meet energy demands during proliferative responses. Producing energy by a high rate of cellular glycolysis even in the presence of oxygen is termed the Warburg effect and was first described in cancer cells [100] and is now used to detect aberrant metabolism of early tumors by positron-emission scanning.

It is also well known that mammalian Target of Rapamycin (mTOR), crucially needed for cell growth, proliferation and motility, is modulated by intracellular ATP concentrations [101]. Rapamycin has potent effects boosting Treg cell induction and functions [102] and divergent manifestations of Treg cell anergy *in vitro* and *in vivo* might be explained by

“oscillatory signals” mediated by leptin-mTOR interactions [103]. Whether Treg cells exert immunosuppressive functions on target cells that are dependent upon CD39-dependent scavenging of extracellular nucleotides and conversion to nucleosides for salvage, which in turn modulate mTOR and other such crucial pathways in activated T cells, remains to be investigated.

Cytolytic molecules

In addition to regulation by suppression, Treg cells can also mediate their activity by killing effector cells. This process involves the release of cytolytic molecules, such as granzyme A/B, perforin or through the TRAIL-DR5 (tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5) pathway [8]. In a reciprocal manner, increased cytotoxicity afforded by granzyme B/perforin expression by CD4⁺ invariant NKT cells against Treg appears to contribute to immunoallergic diseases [104].

Granzyme molecules are highly expressed in Treg cells and deficiency further inhibits Treg cell function. Moreover, target cells that over-express a specific inhibitor of granzyme are resistant to death by apoptosis [8]. Similarly, increased expression of the cytolytic galectin-1 has been observed in both human and mouse Treg cells, and Treg cells isolated from galectin-1-deficient mice show impaired activity *in vitro* [8].

Membrane-associated molecules that down-modulate the activation of effector cells

The expression of a number of inhibitory membrane-associated molecules that can down-modulate effector cell function has also been implicated in Treg-mediated suppression. The most characterized surface molecule expressed by Treg cells is the negative regulator CTLA-4, which plays a critical role in the function of Treg cells [76]. Treg-derived CTLA-4 mediates its activities through interaction with the co-stimulatory molecules CD80 and CD86 on DC, leading to down-regulation of the maturation markers or induction of the potent suppressive enzyme IDO in the antigen presenting cells, and subsequent suppression of T cell activation and function [76].

CTLA-4 may also directly suppress effector T cells by binding to the accessory molecules, which are expressed on the cells upon activation. Furthermore, it has also been suggested that signaling via CTLA-4 transduces a co-stimulatory signal to Treg cells which is required for their activation. CTLA-4-deficient mice develop severe autoimmune disease, however, Treg cells isolated from these mice display normal regulatory function *in vitro* [76]. Curiously, CD39-generated adenosine also induces PD1 and CTLA-4 on T effector cells which further integrates these Treg cell mechanisms [105].

Recent studies have identified another inhibitory membrane-associated molecule on Treg cells known as lymphocyte-activation gene 3 (LAG-3) [106]. LAG-3 is a CD4-related adhesion protein that binds to MHC class II molecules expressed on DC and other antigen presenting cells. Binding of LAG-3 to MHCII molecules induces a negative signal that inhibits DC maturation and function. Antibodies against LAG-3 block Treg cell suppression *in vitro* and *in vivo*, and Treg cells from LAG-3-deficient mice exhibit reduced function *in vitro*. However, loss of LAG-3 in mice does not result in the development of autoimmunity [106].

Regulation of Treg cell function

As over-suppression mediated by Treg cells could hinder the ability to elicit an effective immune response to microbes and tumor cells, control of the magnitude of Treg cell activity

is crucial [76;94]. Indeed, during an inflammatory response associated with pathogenic infection or cancer, a number of inflammatory cytokines (such as IL-6 and TNF- α) produced by mature DC have inhibitory effects on the activation and expansion of Treg cells. Also, the binding of microbial particles to TLR (TLR8 and TLR2) expressed by Treg cells triggers inhibitory signals in these cells. Lastly, in response to inflammatory cytokines, strong TCR activation and GITR stimulation, effector memory T cells may become resistant to Treg-mediated suppression [76].

The inhibition of Treg function together with the resistance of effector T cells to suppression permits the development of an efficient immunity during the inflammatory response. The molecular mechanisms and basis for these observations remains unclear at this time.

Concluding remarks

Multiple mechanisms have been proposed to attempt to explain how Treg cells inhibit CD4⁺ and other effector T cells. Treg can exert their effects through direct cell contact or through production of cytokines. It is clear that in some cases Treg cells can exert their effects through modulation of APC maturation; inhibition of activation and differentiation of T cells and induction of apoptosis. The large diversity of molecular mechanisms of Treg cells may be secondary to the broad spectrum of pathogens (bacteria, viruses, parasites, allo antigens) that we encounter.

The data presented here suggest that the relative contributions of many putative Treg-associated molecules to the suppressive activity of the cells may depend on various factors. These might include the genetic background of the host species, the stimulant (allo)antigen, experimental system, and the site and type of the immune response. Many presumed inhibitory pathways might intersect e.g. IDO and CD39, CTLA-4 and adenosine and be synergistic or complementary.

Dissecting the contribution of Treg cell molecules in these different settings is a key to the future of Treg cell research, which may help explain the contradictory results obtained from the *in vitro* and *in vivo* studies. Ultimately, a better understanding of how Treg cells work and defining the molecules these unusual and fascinating cells employ could potentially lead to the development of novel and possibly safer therapeutic approaches in transplantation and for a wide range of immune-mediated diseases.

List of Abbreviations

APC	Antigen presenting cells
BM	Bone marrow
BMT	Bone-marrow transplantation
cAMP	Cyclic AMP
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cells
DN	Double negative Treg cells
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cells

FcR	Fc receptor
FITC	Fluorescein isothiocyanate
FGL2	Fibrinogen-Like Protein 2
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GVHD	Graft-versus-host-disease
H&E	Hematoxylin and Eosin
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
IBD	Inflammatory Bowel Disease
IDO	Indoleamine 2,3-dioxygenase
IEL	Intestinal intraepithelial lymphocyte
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
LAG-3	Lymphocyte activation gene-3
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MS	Multiple sclerosis
mTOR	Mammalian Target of Rapamycin
NAD	Nicotinamide adenine dinucleotide
NKT	Natural killer T cells
PAMP	Pathogen associated molecular patterns
SCID	Severe combined immunodeficiency
TCR	T cell receptor
TGF-β	Tumor growth factor
Th3	T helper type 3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL-DR5	Tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5
Treg	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Regulatory T cells
Tr1	Regulatory T cells type 1

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Figure 1. Natural and adaptive regulatory T cells

Several different types of regulatory T cells exist which are classified into two major subgroups, natural regulatory T cells produced by the thymus and adaptive regulatory T cells that are induced in the periphery upon antigenic stimulation of naive T cells under tolerogenic conditions (such as TGF- β , IL-10 and immature DC). Thymus-derived CD4⁺CD25⁺Foxp3⁺ Treg cells, DN and some subsets of CD8 suppressor cells can also develop in the periphery. Abbreviations: nTreg- naturally occurring CD4⁺CD25⁺Foxp3⁺ Treg cells; iTreg- induced CD4⁺CD25⁺Foxp3⁺ Treg cells; NKT- natural killer T cells; DN- double negative Treg cells; Th3- T helper type 3; Tr1- type 1 regulatory T cells.

Figure 2. Reduction or increase in Treg cell numbers/activity is implicated in various pathologies
Reduction in Treg cell numbers or activity results in the development of autoimmunity, allergy and graft rejection. Increases in Treg cell numbers or aberrant function may cause susceptibility to chronic infection and predispose to tumor development.

Table shows some of the main Treg-associated molecules that have been described in the literature

Deletion of most of the effector genes leads to the development (to some degree) of autoimmune diseases. Treg cells from the respective gene-deficient mice, however, in some cases exhibit normal suppressive activity *in vitro*. Similarly, antibodies to the respective proteins block Treg cell activity *in vivo*, but in some occasions fail to do so *in vitro*. Further work is required to fully understand the controversy between the *in vivo* and *in vitro* results.

Gene	Treg-associated molecules					
	KO mice Autoimmunity developments	KO mice Treg function (<i>in vitro</i>)	KO mice Treg function (<i>in vivo</i>)	KO mice Treg numbers	Blocking antibody Effect on Treg (<i>in vivo</i>)	Blocking antibody Effect on Treg (<i>in vitro</i>)
Foxp3	+	–	–	–	N/A	N/A
IL-2	+	Normal	N/A	↓	+	N/A
CD25	+	Normal	N/A	↓	+	N/A
CTLA-4	+	Normal	Normal	↑	+	–
TGF-β	+	Normal	↓	↓	+	–
IL-10	Colitis in dirty environment	Normal	↓	N/A	+	–
CD39	+	↓	↓	N/A	N/A	N/A
LAG-3	–	↓	↓	N/A	+	+
Galectin-1	Susceptibility to induced-EAE	↓	N/A	N/A	N/A	+
GTR	N/A	Normal	N/A	N/A	+	+
FGL2	In aged mice	↓	N/A	↑	N/A	+

N/A: not applicable; +: positive; -: negative/none

Table 2

Based on their molecular characterization and function, effector molecules of Treg cells can be classified into four groups: immunosuppressive cytokines, molecules involved in metabolic signaling, cytolytic molecules and membrane-associated molecules that down-modulate the activation of effector cells.

Immunosuppressive Cytokines	Metabolic Signaling	Cytolytic Molecules	Membrane-associated Molecules
TGF- β	cAMP	Granzyme A/B	CTLA-4
IL-10	CD39	Perforin	LAG-3
IL-35	CD73	TRAIL-DR5	
FGL2	NAD	Galectin-1	

Abbreviations: IL- interleukin; TGF- β - Tumor growth factor β ; FGL2- Fibrinogen-like protein; cAMP-inhibitory second messenger cyclic AMP; NAD- nicotinamide adenine dinucleotide as metabolized by CD38 and operative at P2X7; TRAIL-DR5 (tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5); CTLA-4- Cytotoxic T-Lymphocyte Antigen 4; LAG-3- Lymphocyte-activation gene 3.