

REVIEW

Regulating regulatory T cells

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Regulatory T cells (Tregs) are a specialized subpopulation of T cells that act to suppress activation of other immune cells and thereby maintain immune system homeostasis, self-tolerance as well as control excessive response to foreign antigens. The mere concept of Tregs was the subject of significant controversy among immunologists for many years owing to the paucity of reliable markers for defining these cells and the ambiguity of the nature and molecular basis of suppressive phenomena. However, recent advances in the molecular characterization of this cell population have firmly established their existence and their vital role in the vertebrate immune system. Of interest, accumulating evidence from both humans and experimental animal models has implicated the involvement of Tregs in the development of graft-versus-host disease (GVHD). The demonstration that Tregs could separate GVHD from graft-versus-tumor (GVT) activity suggests that their immunosuppressive potential could be manipulated to reduce GVHD without detrimental consequence on GVT effect. Although a variety of T lymphocytes with suppressive capabilities have been reported, the two best-characterized subsets are the naturally arising, intrathymic-generated Tregs (natural Tregs) and the peripherally generated, inducible Tregs (inducible Tregs). This review summarizes our current knowledge of the generation, function and regulation of these two populations of Tregs during an immune response. Their role in the development of GVHD and their therapeutic potential for the prevention and treatment of GVHD will also be described.

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Introduction

One of the immune system's main functions is to inhibit potentially harmful immune responses against self-antigens. The T-cell repertoire is shaped by both positive and negative selection in the thymus.¹ The developing T cells, each with a different T-cell receptor (TCR), undergo a positive selection step where only T cells that can interact with the major histocompatibility complex (MHC) survive. This is followed by a negative selection process, where T cells with high avidity for self-MHC are deleted. As a result, only T cells that can interact with MHC with relatively low avidity survive and migrate out to the periphery. Although a major mechanism of immunologic self-tolerance is negative selection of self-reactive T cells in the thymus, experiments in the T-cell compartment have also suggested that 'receptor editing' and 'clonal anergy' are involved in tolerance induction.^{2–4} Although these cell intrinsic processes are essential for survival of the organism, they are imperfect at times and autoreactive T cells can be found in the peripheral blood of immunologically competent animals⁵ and humans.⁶ To keep these potentially hazardous autoreactive T cells under control, the immune system has evolved an extrinsic form of dominant tolerance which involves regulatory T cells (Tregs). Besides their critical role in inducing self-tolerance, Tregs are also involved in controlling the immune responses to a myriad of foreign agents including infectious microorganisms⁷ and alloantigens.⁸

An important question in the field of immunology is how the immunosuppressive activity of Tregs is induced and modulated during the course of an immune response. Failure of immunologic self-tolerance frequently leads to the development of autoimmune disease. On the other hand, although an effective, robust immune response is desirable against potentially pathogenic agents, uncontrolled response may lead to undesirable tissue damage.

In this review, we shall focus on recent progress in our understanding of the regulation and function of Tregs in controlling physiological and pathological immune responses. We shall also describe briefly the role of Tregs in the development of graft-versus-host disease (GVHD) and efforts to exploit their therapeutic potential for the prophylaxis and treatment of GVHD.

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Molecular characterization of Tregs

Studies of Tregs have identified multiple populations of cells with immunosuppressive capabilities but with different cell surface markers, site and mode of generation (Table 1). The best-described population of Tregs is the so-called natural Tregs.⁹ These are CD4⁺ T cells arising during T-cell development in the thymus and constitutively expressing the α chain of the interleukin (IL)-2 receptor (CD25). In addition to natural Tregs, other types of Tregs can be induced (inducible Tregs) from naïve T cells in the periphery during the course of a normal immune response. There are at least two populations of inducible Tregs: Th3 cells and Tr1 cells. Th3 cells were first identified because of their role in oral tolerance, through the secretion of transforming growth factor (TGF)- β .¹⁰ Tr1 cells are very similar to Th3 cells but secrete large amount of IL-10^{11,12} and were characterized on the basis of their role in preventing autoimmune colitis.¹³ Although the inducible Tregs are largely contained within the CD4⁺ T-cell compartment, they are distinct from the natural Tregs in various aspects. Other T-cell populations with demonstrable immunosuppressive function, such as CD8⁺ Tregs^{14,15} and natural killer Tregs,¹⁶ have also been reported in different models of autoimmune diseases and transplantation tolerance.

Natural Tregs

Naturally occurring CD4⁺ Tregs constitutively express a variety of cell surface molecules commonly associated with activated/memory cell phenotype. These include CD25, CD45RB^{low}, CD62L, cytotoxic T-lymphocyte antigen-4 (CTLA-4, or CD152) and glucocorticoid-induced tumor necrosis factor receptor (GITR) family-related gene. Although none of these surface markers is uniquely expressed by natural Tregs, their level of expression and constitutive nature have made them useful as functional descriptors and enabled the consistent isolation and

investigation of CD4⁺ T cells with suppressive capabilities. Before the identification of the forkhead family transcription factor FoxP3 (see below), expression of the two cell surface molecules, CD4 and CD25, was used to define this population of Tregs. Thus, these cells were often referred to as CD4⁺CD25⁺ Tregs.

Work in recent years has shown that the forkhead family transcription factor FoxP3 is critically important for the development and function of Tregs.¹⁷ Genetic mutations in the gene encoding FoxP3 have been identified in both humans and mice, and result in fatal autoimmune diseases. Humans with mutations in FoxP3 suffer from a severe and rapidly fatal autoimmune disorder known as the immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome. The IPEX syndrome is characterized by the development of overwhelming systemic autoimmunity in the first year of life, resulting in the commonly observed triad of watery diarrhea, eczematous dermatitis and insulin-dependent diabetes mellitus. The majority of the affected males die during the first year of life of either metabolic derangements or sepsis. An analogous disease also occurs in mice, known as 'scurfy', owing to a spontaneous mutation in FoxP3. Furthermore, ectopic expression of FoxP3 can phenotypically and functionally convert effector T cells (Teffs) to Tregs.¹⁷ It appears that in mice, FoxP3 expression is both necessary and sufficient for Treg development. However, there is accumulating evidence demonstrating that, in contrast to mice, human FoxP3 is transiently expressed in activated T cells where it could be detected within 24 h and peaked at 72 h.¹⁷ The observation that abundant FoxP3 messenger RNA (mRNA) was detected in the recently activated CD4⁺CD25⁺ cells lacking regulatory function¹⁸ strongly suggests that in humans, FoxP3 expression alone is not sufficient to indicate regulatory activity of CD4⁺CD25⁺ cells.

The quest to identify molecules, especially cell surface markers that uniquely define Tregs, has led to a recent identification of an additional potential marker. Two studies have reported an inverse correlation between the

Table 1 Type of Treg subsets in the immune system

Treg subset	Experimental model	Phenotype	Generation	References
Natural Treg	Mouse	CD4 ⁺ CD25 ⁺	Intrathymus Mechanism Positive selection by TE Lack of negative selection by TE Positive selection by HC	26–32 26–29 30,31 32
Th3	Mouse oral tolerance	CD4 ⁺ , TGF- β -secreting	Oral administration of low-dose antigen	10
Tr1	Human/mouse <i>in vitro</i> Human <i>in vitro</i> Mouse colitis	CD4 ⁺ , IL-10-secreting CD4 ⁺ , IL-10-/IFN- γ -secreting CD4 ⁺ , IL-10-secreting	Vitamin D3 and dexamethasone Naïve CD4 ⁺ T cells in IL-10 and IFN- α Chronic activation of CD4 ⁺ T cells in IL-10	11 12 13
CD8 ⁺ Treg	Human <i>in vitro</i> Rat oral tolerance	CD8 ⁺ , IL-10-secreting CD8 ⁺ , increased IL-4 mRNA	Naïve CD8 ⁺ T cells primed with CD40-activated plasmacytoid DC ND	15 14
NK Treg	Mouse transplantation	V α 14 NKT cells	V α 14/V β 8.2 TCR in RAG-1-deficient mice	16

Abbreviations: DC = dendritic cell; HC = hematopoietic-derived cell; IFN = interferon; IL = interleukin; ND = not done; NKT = natural killer T; Treg = regulatory T cell; TCR = T-cell receptor; TE = thymic epithelial; TGF = transforming growth factor.

expression of the IL-7 receptor α chain (CD127) and the suppressive function of the CD4⁺FoxP3-expressing T cells.^{19,20} It is important to note that CD127 is down-regulated in all human T cells after activation. However, although CD127 is reportedly re-expressed on the majority of effector and memory T cells,^{21–23} it remains low or even undetectable in CD4⁺ FoxP3-expressing T cells.^{19,20} Liu *et al.*¹⁹ further demonstrated that cells separated based solely on CD4 and CD127 expression were anergic and, although accounting for significantly more cells compared to previous methods using other cell surface markers, had comparable suppressive capabilities to the ‘classic’ CD4⁺CD25^{high} T-cell subset.

Inducible Tregs

The inducible Tregs are induced from naïve T cells in the periphery under low-dose antigenic stimulation,²⁴ or by immunosuppressive cytokines such as TGF- β .²⁵ They do not have the classical CD4⁺CD25⁺ T-cell phenotype and are rather defined on the basis of their cytokine profile. As is the case with natural Tregs, there is no unique cell surface marker to distinguish them from other T-cell subsets. Although FoxP3 is expressed by natural Tregs, at present it is not clear whether FoxP3 also regulates the development of either Th3 or Tr1 cells. However, TGF- β , a cytokine mediator of Th3 cells, has been shown to convert peripheral CD4⁺CD25⁻ naïve T cells into regulatory CD4⁺CD25⁺ T cells, presumably through the induction of FoxP3 expression.²⁵

Generation of Tregs

Natural Tregs

Natural Tregs develop in the thymus where they appear to be positively selected on the cortical medullary epithelial cells.²⁶ It has been observed in TCR-transgenic mice that co-expressing of agonist ligands in radioresistant tissues, specifically in thymic epithelial (TE) cells, results in increased number of CD4⁺CD25⁺ T cells.²⁷ This increase is mostly due to *de novo* generation of CD4⁺CD25⁺ thymocytes as their absolute number is increased by 10–30 fold in various transgenic models.^{27–29} Selection of CD4⁺CD25⁺ thymocytes appears to require a TCR with intermediate affinity for self-peptide because thymocytes expressing TCRs with low affinity do not undergo selection by this pathway.²⁸ The results from other studies, however, suggest that a ‘defective’ negative selection by TE cells may contribute to enriched autoreactive cells in the Treg repertoire because these precursors appear resistant to clonal deletion induced by ligands expressed by TE.^{30,31} For the moment, it remains unclear if the observed enrichment of self-reactivity of the Treg repertoire is due to positive selection by self-ligand or to lack of negative selection by TE or both. In addition, analysis of the frequency of a number of superantigen-reactive V β families in mice showed that CD4⁺CD25⁺ thymocytes are as susceptible to endogenous superantigen-mediated clonal deletion as CD4⁺CD25⁻ T cells.³² As TE cells do not express superantigens, these results suggest that TE-selected Tregs are subjected to negative selection by hematopoietic-derived cells.

Inducible Tregs

In contrast to the intrathymic generating of natural Tregs, inducible Tregs are generated in the periphery, presumably to help terminate the response when the pathogen is eliminated and to prevent secondary autoimmunity. Their generation is dependent on the peripheral factors such as the maturation and type of the stimulating antigen-presenting cells (APC),^{33,34} availability of cytokines such as TGF- β ²⁵ and the presence of low-dose antigens.²⁴ The differentiation of inducible Tregs appears to be controlled, at least under certain condition, by immature dendritic cells (DCs) because repetitive stimulation of naïve T cells with immature DCs resulted in the development of T cells with suppressive function.³³ However, it has also been demonstrated that particular DC subsets can induce a Treg phenotype irrespective of their maturation state.³⁴

Furthermore, studies of Tregs in transplantation tolerance have led to the proposal that natural Tregs may play a role in inducing the inducible Tregs, through a process known as ‘linked suppression’.^{35–37} In this model, ‘linked suppression’ occurs when a potentially alloreactive T cell comes under the influence of a Treg as both cells recognize their respective alloantigens presented by the same APC.^{35,36} During this ‘re-education’ process, the potentially hazardous alloreactive T cell is converted into a Treg and, in turn, can induce other CD4⁺ T cells into the regulatory phenotype, thus propagating the tolerant state. The secretion of IL-10 and TGF- β by inducible Tregs and their effect, either directly on the naïve T cells or on the stimulating APC, have been implicated in this process.

Requirements for *in vivo* suppression

Activation

The activation of natural Tregs appears to require antigen specificities. Using TCR-transgenic mice specific for ovalbumin (OVA), it was shown that adequate protection from GVHD was obtained only when the mice were immunized with the antigen recognized by the host T cells which include Tregs.³⁸ Furthermore, Tregs are significantly more effective in controlling GVHD when *ex vivo* expanded with host APC compared with third-party APC.³⁹ This result is most consistent with the assumption that *ex vivo* expansion of Tregs using host APC leads to better activation of host antigen-specific Tregs *in vivo*.

Tregs have also been shown to recognize antigens derived from pathogens and such recognition is an essential step in their regulatory function. Tregs from mice chronically infected with either schistosoma or leishmania can produce IL-10 in response to the same parasite antigens but not to other stimuli or pathogens.^{40,41} The most convincing data actually came from human studies. CD4⁺CD25⁺ Tregs from the peripheral blood of asymptomatic human immunodeficiency virus-infected individuals mediate immunosuppression in an antigen-specific manner.⁴² Similarly, in patients infected with *Helicobacter pylori*, Treg-mediated suppression could be shown only with *H. pylori* antigens.⁴³

Other requirements for *in vivo* suppression: homing, expansion and migration to inflamed tissues

Unlike the situation *in vitro*, there is ample space *in vivo* for the activated Teffs to evade suppression unless Tregs are able to come into close contact with Teffs with a given antigenic specificity. This requirement dictates that Tregs must be able to localize to various part of the body where antigenic stimulation takes place, beginning in the draining lymph nodes. Additionally, *in vitro* studies have consistently demonstrated that meaningful suppression of activated T cells could only be achieved with at least a 1:3 ratio of Tregs to Teffs; lower ratios resulted in little or no suppression. As Tregs are accounted for only 5–10% of peripheral CD4⁺ T cells and 3% of total T cells *in vivo*, a combination of selective homing and/or expansion must occur *in vivo* for Tregs to mount an effective immunosuppressive effect. Indeed, in nonobese diabetic (NOD) mouse models, only suppressor T cells that were able to home to and proliferate in pancreatic lymph nodes by recognizing agonist ligands were capable of preventing and suppressing the disease after transfer into NOD mice. Cells with similar suppressor potentials *in vitro* but which were unable to accumulate and proliferate in the draining lymph nodes were ineffective in disease prevention and suppression.^{44–46} Thus, it seems that *in vivo*, the specific homing to and proliferation in the draining lymph nodes represent essential features that enable Tregs to effectively suppress the early phase of an immune response.

The ability of Tregs to suppress activation of other immune cells is not restricted to inhibition of their early proliferation but also involves suppression of effector cells at the targeted sites. This, in turn, requires Tregs to migrate toward sites of inflammation. Indeed, activated Tregs have been shown to change their homing receptors which permit them to exit into the inflamed tissue and suppress the activated Teffs.⁴⁷ One study demonstrated that Tr1 cells tend to migrate toward the inflamed sites whereas natural Tregs are predominantly located in the lymphoid organs.⁴⁸ The results from other studies, however, established the existence of CD4⁺CD25⁺ Tregs within the tolerated allografts.⁴⁹

Mechanism of suppression: soluble cytokines versus direct cell–cell interaction

Although Tregs require antigen exposure to initiate suppressive activity, *in vitro* studies revealed that once

activated, Tregs inhibit immune response in an antigen-nonspecific manner.⁵⁰ In addition, in the OVA-specific TCR-transgenic mice, it was shown that a FoxP3-transduced CD4⁺ T cell clone specific for a single host-antigen (OVA) efficiently protected the host from GVHD.³⁸ This result is most readily explained by antigen specificity of the activation phase and bystander suppression during the effector suppressor phase.

The exact mechanism of suppression, however, remains elusive with differences between *in vitro* and *in vivo* results and between different animal models regarding the relative contribution of soluble cytokines and direct cell–cell contact (Table 2). The results from several *in vitro* studies suggest that CD4⁺CD25⁺ Tregs suppress Teff proliferation through a cell-contact dependent and cytokine independent mechanism.^{51–53} In particular, they implicate a role for the accessory molecules such as CTLA-4 and GITR expressed on the surface of Tregs.^{54,55} The involvement of CTLA-4 and its ligands, CD80 and CD86, in Treg-mediated suppression is further supported by the results obtained from a murine GVHD model.⁵⁶ GVHD develops following the injection of CD4⁺CD25[−] T cells, either wild type or CD80-deficient or CD86-deficient, into lymphopenic animals deficient in recombination-activating gene 2. Co-injection of CD4⁺CD25⁺ T cells with CD4⁺CD25[−] T cells prevents disease caused by wild-type Teffs but not by Teffs deficient in either CD80 or CD86. The results support a signaling model in which suppression of T-cell activation requires binding of CD80 and CD86 on the activated T cells to CTLA-4 on Tregs. In addition to CTLA-4 and GITR, cell surface TGF- β 1 has also been implicated in mediating the immunosuppressive effect of Tregs.⁵⁷

In contrast to natural Tregs, the inducible Tregs, including both Th3 and Tr1 cells, appear to function independently of cell–cell interaction and suppress immune responses through the secretion of cytokines such as IL-10⁵⁸ and TGF- β .⁵⁹ The involvement of these immunosuppressive cytokines in dampening the immune responses is further supported by *in vivo* models which demonstrated that blockade of either IL-10 or TGF- β abrogated Treg-mediated unresponsiveness to alloantigens.^{58,59} Perhaps, the model of ‘linked suppression’ which involves both direct cell contact and soluble cytokines could reconcile some of the apparent disparities regarding the mechanisms of suppression by Tregs.

Table 2 Mechanism of suppression: natural Tregs versus inducible Tregs

Treg subset	Immunophenotype	Mechanism of suppression <i>in vitro</i>	Mechanism of suppression <i>in vivo</i>	References
Natural Tregs	CD4 ⁺ CD25 ⁺	Cell–cell contact	ND	51
		ND	IL-10 ^a	52
		CTLA-4	CTLA-4	54,56
		GITR	GITR	55
		Cell–cell contact, cell surface TGF- β	ND	57
Inducible Tregs	CD4 ⁺ , IL-10-secreting	IL-10	IL-10	58
	CD4 ⁺ , TGF- β -secreting	TGF- β	TGF- β	59

Abbreviations: CTLA-4 = cytotoxic T-lymphocyte antigen-4; GITR = glucocorticoid-induced tumor necrosis factor receptor; IL = interleukin; ND = not done; Treg = regulatory T cell; TGF = transforming growth factor.

^aIn colitis but not gastritis.

Downregulation and/or inactivation

Little is known as to how the immunosuppressive activity of Tregs is downregulated and/or terminated once it is no longer beneficial to the host. It appears that CD4⁺CD25⁺ Tregs committed to suppression after antigenic stimulation can survive for long periods of time without cell division and in the absence of the antigen that induced their formation.^{60,61} These cells can recirculate, like naïve T cells, from peripheral blood to antigen-draining lymph nodes where they can accumulate through cell division at roughly the same rate as naïve T cells.^{38,62,63} As Tregs are intrinsically hypoproliferative and hyporesponsive, it is reasonable to propose that once the inflammatory 'cytokine storm' subsides and in the absence of antigenic stimulation, Tregs will resume to their original anergic state with diminishing immunosuppressive activity. Further studies are certainly needed to address this important issue.

Tregs in hematopoietic cell transplantation

Allogeneic hematopoietic cell transplantation (alloHCT) is now widely used in the treatment of various hematologic malignancies, solid tumors and inherited diseases. The lack of well-matched donors, however, restricts the number and hampers the success of such transplants. Mismatched alloHCT carries a high risk of life-threatening GVHD owing to activation of donor immune cells, especially T lymphocytes, by antigens present on normal host cells. Removal of donor mature T cells can prevent GVHD but leads to a delay in immune reconstitution, and thus an increased incidence of opportunistic infections.^{64,65} Moreover, donor T cells have been demonstrated to exert the powerful graft-versus-tumor (GVT) effect which is essential in controlling disease relapse.⁶⁶ Balancing GVHD and GVT and anti-pathogen effects has been the challenge in the field of hematopoietic transplantation immunology for decades. Recent knowledge in the studies of Treg has implicated their involvement in the development of GVHD and suggested that the immunosuppressive potential of Tregs can be harnessed therapeutically to reduce the incidence and/or severity of GVHD.

In animal models

A role for natural Tregs in the development of transplant tolerance was first demonstrated by their ability to suppress GVHD in murine models of alloHCT. Transferring of allogeneic CD4⁺CD25⁻ naïve or Tregs led to GVHD which was significantly delayed by co-transferring of purified CD4⁺CD25⁺ Tregs.⁶⁷ These observations were subsequently confirmed in other experimental models: infusion of purified populations of CD4⁺CD25⁺ T cells together with the graft successfully prevented the development of lethal GVHD.^{39,67-70} A legitimate concern with regards to infusing of Tregs at the time of HCT is that Tregs might inhibit the immune response against tumor cells and therefore decrease the beneficial GVT effects of donor T cells. Edinger *et al.*⁷⁰ addressed this issue by demonstrating

that in host mice with leukemia and lymphoma, infusion of donor CD4⁺CD25⁺ Tregs at the time of HCT could control early expansion of alloreactive T cells without abrogating donor Tregs' GVT function. The animals did not develop lethal GVHD whereas tumor growth was controlled and leukemic cells were eliminated within 2 weeks after transplant. In addition, it has been demonstrated in another model of murine GVHD that the infusion of donor CD4⁺CD25⁺ Tregs at a later time point, with the intention of providing the initial window of time in which the beneficial GVT response is permitted to begin, was effective in controlling ongoing GVHD.⁷¹ Thus, the data accumulated from the animal studies clearly indicate that CD4⁺CD25⁺ Tregs play a vital role in downregulating GVHD. The demonstration that Tregs could separate GVHD from GVT activity suggests that their immunosuppressive potential could be manipulated to reduce GVHD without detrimental consequence on GVT effect.

Tregs in human GVHD

Attempts to investigate the potential role of Tregs in human acute GVHD (aGVHD) have been limited owing to slow immune reconstitution and ongoing inflammatory responses, which further complicate the assessment of immune parameters. One study of patients with acute allogeneic GVHD reported an inverse correlation between FoxP3 expression and the grade of aGVHD.⁷² Expression of FoxP3 mRNA was almost undetectable in patients with grade III-IV aGVHD and significantly reduced (by almost twofolds) in patients with grade I-II aGVHD compared with patients who did not develop clinical GVHD. Interestingly, sequential analysis of peripheral blood lymphocytes from patients with aGVHD evolving into chronic GVHD (cGVHD) revealed that FoxP3 expression was consistently reduced where the disease was active, but returned to normal after resolution of GVHD. Moreover, normalization of FoxP3 expression coincided with *de novo* T-cell development as assessed by T-cell recombination excision circles. Taken together, these data suggest that these Tregs are recent thymic emigrants and play a crucial role in downregulating aGVHD.

Conflicting data have been reported with regards to the role of Tregs in the development of cGVHD in humans (Table 3).⁷³⁻⁷⁷ The first study was performed by Clark *et al.*⁷³ in which they defined Treg as CD4⁺CD25^{high} and used flow cytometry to measure the size of the Treg pool in peripheral blood of 40 patients who survived more than 100 days after alloHCT. The authors reported that patients with cGVHD had a significant increased Tregs, expressed both as a percentage of CD4⁺ T cells or as absolute counts, compared to patients without cGVHD. They also purified Tregs from patients with cGVHD and used *in vitro* functional assay to demonstrate that these cells display suppressive capabilities comparable to Tregs isolated from healthy individuals.

Sanchez *et al.*⁷⁴ conducted similar study on 35 consecutive patients who underwent alloHCT. They found a small, but not statistically significant, increase in the absolute number of CD4⁺CD25^{high} Tregs in patients with cGVHD compared to patients without the disease. The

Table 3 Studies of Tregs in human cGVHD

References	Patient populations	Treg phenotype	Measured parameters	Results
Clark <i>et al.</i> ⁷³	alloHCT (retrospective) cGVHD, <i>n</i> = 17 No cGVHD, <i>n</i> = 23	CD4 ⁺ CD25 ^{high}	% Treg/CD4 ⁺ T cells Absolute number	↑ ↑
Sanchez <i>et al.</i> ⁷⁴	alloHCT (prospective) cGVHD samples, <i>n</i> = 8 Resolved cGVHD samples, <i>n</i> = 27 No cGVHD samples, <i>n</i> = 29	CD4 ⁺ CD25 ^{high}	Absolute number CD134 ⁺ T cells/Tregs	↑ ^a ↑
Miura <i>et al.</i> ⁷⁵	alloHCT (retrospective) cGVHD, <i>n</i> = 28 No cGVHD, <i>n</i> = 8 autoHCT (retrospective) cGVHD, <i>n</i> = 16 No cGVHD, <i>n</i> = 23	CD4 ⁺ CD25 ⁺	FoxP3 mRNA in CD4 ⁺ CD25 ⁺ T cells	↓
Meignin <i>et al.</i> ⁷⁶	alloHCT (prospective) cGVHD, <i>n</i> = 20 No cGVHD, <i>n</i> = 11 Healthy control <i>n</i> = 5	CD4 ⁺ CD25 ^{high}	Absolute number % Treg/CD4 ⁺ T cells FoxP3 mRNA in CD4 ⁺ CD25 ^{high} T cells Absolute number	↓ ^b ↔ ↔ ↔
Zorn <i>et al.</i> ⁷⁷	alloHCT (retrospective) cGVHD, <i>n</i> = 30 No cGVHD, <i>n</i> = 27 Healthy control, <i>n</i> = 26	CD4 ⁺ CD25 ⁺	% Tregs/total lymphs FoxP3 mRNA in PBMC	↓ ↓

Abbreviations: alloHCT = allogeneic hematopoietic cell transplantation; cGVHD = chronic graft-versus-host disease; mRNA = messenger RNA; PBMC = peripheral blood mononuclear cell; Treg = regulatory T cell.

^aNot statistically significant.

^bReduced in alloHCT recipients compared to healthy controls, irrespective of GVHD status.

authors also examined the ratio of activated non-regulatory CD134⁺ (OX40⁺) T cells over CD4⁺CD25^{high} Tregs and found that the CD134⁺/CD25^{high} ratio was remarkably higher in patients with active cGVHD compared to patients without cGVHD or with resolved cGVHD.

The characterization of the forkhead transcription factor, FoxP3, and initial reports that FoxP3 expression was both necessary and sufficient for Treg development have led to the wide use of this intracellular marker in Treg studies. Miura *et al.*⁷⁵ used quantitative polymerase chain reaction (PCR) of peripheral blood mononuclear cells (PBMCs) to examine the expression of various markers for Tregs. FoxP3 mRNA expression was significantly decreased in PBMC from patients with allogeneic GVHD and autologous GVHD compared to patients without GVHD. Moreover, expression of FoxP3 was negatively correlated with the severity of GVHD but positively correlated with recent thymic emigrant. In patients without cGVHD, FoxP3 expression increased with time after transplant to eventually reach normal values, whereas the expression remained low in patients with active cGVHD. These data suggest that poor recovery of Tregs during the post-transplant immune reconstitution might contribute to the development of cGVHD. Alternatively, cGVHD might cause damage to TE cells leading to defective thymic function and resulting in decreased generation of natural Tregs.

Two additional studies further addressed the role of Tregs in human cGVHD using both flow cytometry and quantitative PCR analysis of FoxP3 expression. Meignin

*et al.*⁷⁶ found no significant difference between patients with or without cGVHD, in terms of both absolute FoxP3-expressing CD4⁺CD25^{high} cell counts as well as the frequency of these cells within the CD4⁺ T-cell compartment. Although the level of FoxP3 expression in the CD4⁺CD25^{high} population was comparable between alloHCT patients and healthy individuals, there was a profound and persistent CD4⁺ T-cell lymphopenia in alloHCT recipients compared to normal controls. Zorn *et al.*⁷⁷ examined the expression of FoxP3 in total lymphocytes, rather than in the CD4⁺CD25^{high} T cells, as a method to evaluate the ratio of Tregs relative to other immune cells. They found a significant decrease in the frequencies of Tregs in patients with active cGVHD compared to patients who did not develop the disease or who had resolved disease following treatment. However, Seidel *et al.*¹⁸ recently reported a study in 28 pediatric HCT patients monitoring for the development of GVHD in both acute and chronic forms. They found that the levels of FoxP3 expression in CD4⁺CD25^{high} cells were identical in healthy controls and in HCT patients irrespective of GVHD status. It is possible that the apparent discrepancies between these studies simply reflect the varying strategies employed to measure the Treg population including its phenotypic definition. Utilization of additional cell surface marker such as CD127 may help to resolve these contradicting results.

Some investigators have proposed the use of the frequency of Tregs relative to T effs under their immunosuppressive control as a measure of Treg activity *in vivo*.⁷⁸

Collectively, the results obtained so far appear to support the view that Tregs/Teffs balance tips toward Teff in patients with GVHD compared to patients without the disease. Thus, interventions that lead to enhanced function or number of Tregs *in vivo* could help to control or to treat GVHD.

Future directions

Abundant evidence now strongly supports the existence of the once controversial Tregs as major controllers of various physiological and pathological immune responses, and thus a new target for therapeutic intervention. With regard to protection from GVHD, multiple approaches have been designed to enhance Tregs immunosuppressive capability *in vivo*. These include the use of *ex vivo* expanded Tregs, cytokine therapy such as IL-2, selective immunosuppression with agents such as rapamycin, and even extra corporeal photopheresis.⁷⁸ The remarkable results obtained with *ex vivo* expanded Tregs in experimental models are worth noting. Infusion of *ex vivo* activated and expanded Tregs significantly inhibited lethal GVHD in several murine models.^{39,68,71,79} Furthermore, in some cases, the *ex vivo* expanded Tregs appeared to be better than freshly purified CD4⁺CD25⁺ T cells in preventing GVHD.^{71,79} Given these promising results, considerable efforts have been made to expand human Tregs for use in clinical setting. Large-scale expansion of CD4⁺CD25⁺ Tregs, up to 1000 times, using anti-CD3 and anti-CD28 microbeads and high doses of IL-2 has demonstrated the feasibility of bringing this new treatment modality to the bedside.^{80,81} Indeed, several clinical trials using Tregs that have been *ex vivo* expanded by different methods have been initiated.⁸² It is important to note, however, that the number of Tregs required for adequate control of GVHD may be much fewer compared to their need in other clinical settings given the overall lymphopenic condition of HCT patients. The optimal timing of Treg infusion in humans also needs to be determined. Infusion of Tregs together with the transplant might reduce the incidence of aGVHD, as demonstrated in animal models, but early infusion might be more complicated in humans owing to the presence of intense inflammatory responses immediately after transplant and the routine use of immunosuppressive agents for GVHD prophylaxis. Nevertheless, with heightened interest in the field, as it has been for the last few years, we firmly believe that cellular therapies involving Tregs will soon be available to treat various human pathological conditions including GVHD.

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