Regulatory T-cell immunotherapy for allogeneic hematopoietic stem-cell transplantation

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Abstract: From mouse studies to recently published clinical trials, evidence has accumulated on the potential use of regulatory T cells (Treg) in preventing and treating graft-*versus*-host disease following hematopoietic-cell transplantation (HCT). However, controversies remain as to the phenotype and stability of various Treg subsets and their respective roles *in vivo*, the requirement of antigen-specificity of Treg to reduce promiscuous suppression, and the molecular mechanisms by which Treg suppress, particularly in humans. In this review, we discuss recent findings that support a heterogeneous population of human Treg, address advances in understanding how Treg function in the context of HCT, and present data on recent clinical trials that highlight the feasibility and limitations on Treg immunotherapy for graft-*versus*-host disease.

Keywords: graft-*versus*-host disease, hematopoietic-cell transplantation, regulatory T cells

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Introduction

Regulatory T cells (Treg) express the Forkhead box P3 (FOXP3) transcription factor and are indispensable for self-tolerance and the regulation of pathogenic immune responses [Sakaguchi *et al*. 2008]. They mainly do this by suppressing the proliferation and function of effector T cells [Vignali *et al*. 2008]. Given their ability to maintain and restore homeostasis in the immune system, Treg have the potential to ameliorate aberrant immune conditions such as autoimmunity and graft-*versus*-host disease (GvHD). It is feasible to isolate Treg based on key cell surface markers and to expand them to clinically relevant numbers. Administration of these cells in the murine model reduces GvHD, but their efficacy in humans is still uncertain. Correlative human studies to evaluate the association between *in vivo* Treg numbers and the incidence of GvHD have yielded varying results, although most studies suggest an inverse correlation between Treg numbers and GvHD (see Table 1). Recent clinical trials demonstrate the feasibility and general safety of adoptive transfer of Treg, but efficacy outcomes of GvHD and tumor immunity require further investigation. In this review, we provide insights into recent advances on the biology of Treg and discuss how these findings may impact the use of Treg in immunotherapy for GvHD.

Characterization of natural regulatory T cells

CD25

Sakaguchi and colleagues reported constitutive expression of a surface marker, CD25 (IL2Ralpha), on a subset of CD4 T cells that are suppressive [Sakaguchi *et al*. 1995]. These cells comprise approximately 1–10% of peripheral CD4 T cells in mice and humans. The level of CD25 expression correlates with suppressor function, with the top 5–10% representing the majority of the regulatory T cells. Hence, most early studies identified Treg as $CD4CD25^{hi}$ T cells, although the level of CD25 expression used to characterize or purify Treg is variable between studies, making comparisons of data and results less reliable. Nevertheless, $CD4CD25^{hi}$ T cells prevented and reduced autoimmunity, organ transplant rejection, and GvHD [Sakaguchi *et al*. 2008]. However, because T cells express CD25 upon activation, distinguishing regulatory T cells

Table 1. Correlation between Treg numbers and incidence and severity of graft-*versus*-host disease.

Sample	Result	PubMed ID [Reference]
CORRELATIVE RESULTS		
CD4+CD25^{hi} cells from PB at day 30	Treg:CD4+ ratio 3.6X less in GvHD *versus* non-GvHD	20859740 [Ukena *et al.* 2011]
CD4+CD25^{hi} FOXP3+ cells in PB at onset of GvHD	Treg:CD4+ ratio 40% less in GvHD *versus* no GvHD	20302964 [Magenau *et al.* 2010]
CD4+CD25+CD127^{lo}FoxP3+ cells from PB at 3–35 months PT	Treg:CD4+ ratio 38% and 24% less in aGvHD and cGvHD, respectively; ratio 73% lower in aGvHD grade III–IV *versus* aGvHD grade I	20409584 [Li *et al.* 2010]
CD4+CD25$^{int\text{-}hi}$CD127^{lo} cells from PB for first year PT	77% with low Treg:CD4+ ratio developed GvHD; 29% with high Treg:CD4+ ratio developed GvHD	20389017 [Matsuoka *et al.* 2010]
CD4+CD25+FOXP3+ CD62L+ cells from graft	Graft with $>1.1 \times 10^6$/kg Treg lead to better survival from GvHD	19919293 [Vela-Ojeda *et al.* 2010]
CD3+CD4+ CD25^{hi}FOXP3+ from gut, skin and PB during HCT	5 × greater Treg count in GvHD *versus* non-GvHD	20484086 [Ratajczak *et al.* 2010]
CD4+CD25^{hi}FOXP3+ cells from skin, gut, and PB 1 month PT	Treg:CD4+ ratio 70% less in GvHD *versus* non-GvHD	19491336 [Matthews *et al.* 2009]
CD4+CD25+FOXP3+ from skin at onset of GvHD	Treg:CD3+ ratio 46% and 28% less in aGvHD and cGvHD, respectively	19589483 [Fondi *et al.* 2009]
FoxP3+ from skin 14–93 days PT	6 × chance of low absolute Treg count in aGvHD grade I *versus* aGvHD grade II-III	19838066 [Wu *et al.* 2009]
CD4+FOXP3+ cells from graft	76% who received low Treg developed GvHD; 23% receiving high Treg developed the disease	17452902 [Wolf *et al.* 2007]
CD4+CD25+ FOXP3+ cells from colon, gut, and PB at onset	Treg:CD4+ 70% less in GvHD *versus* non-GvHD; CD4+CD25^{hi}:total lymphocyte ratio 50% less in GvHD *versus* non-GvHD	16278306 [Rieger *et al.* 2006]
CD4+FOXP3+ cells from graft and 30–45 days PT	Absolute Treg in graft 38% less in GvHD *versus* non-GvHD; Treg:CD4+ ratio from PB 97% less in GvHD *versus* non-GvHD	16627754 [Rezvani *et al.* 2006]
CD4+CD25+FOXP3+ cells from PB at onset	Treg:CD4+CD25+ ratio 2.6 × less in cGvHD *versus* non-GvHD	15972448 [Zorn *et al.* 2005]
CD4+CD25+FOXP3+ cells from PB at onset	FoxP3+ expression 100% less in GvHD *versus* non-GvHD; expression 5 × greater in aGvHD grade I–II *versus* aGvHD III–IV	15172973 [Miura *et al.* 2004]
CD4+CD25^{hi} cells from PB within 100 days PT	Treg:CD134+ ratio 16 × less in cGvHD *versus* non-GvHD	15327522 [Sanchez *et al.* 2004]
CD4+CD25^{hi} cells from PB after 100 days PT	Treg:CD4+ ratio 2 × greater in cGvHD *versus* non-GvHD	14604970 [Clark *et al.* 2004]
CD4+CD25+ cells from graft	Treg:CD4+ ratio 4 × greater in GvHD *versus* non-GvHD	12907445 [Stanzani *et al.* 2004]
NONCORRELATIVE RESULTS		
CD4+CD25^{hi}FOXP3+ cells from PB for 3 months PT	No correlation	20457268 [Pastore *et al.* 2011]
FOXP3+ cells from PB at onset of GvHD	No correlation	20870026 [Lord *et al.* 2011]
CD4+CD25+FOXP3+ cells from graft	No correlation	20691733 [Vitti *et al.* 2010]
CD4+CD25^{hi}FOXP3+ from skin and PB at day 7, 30, and 60	No correlation	18571003 [Clark *et al.* 2004]
CD4+FOXP3+ cells from PB 1–3 months PT	No correlation	17478639 [Mielke *et al.* 2007]
CD4+FOXP3+ cells from graft	No correlation	17504991 [Pabst *et al.* 2007]
CD4+CD25^{hi}FOXP3+ cells from PB 31 months PT	No correlation	16038781 [Meignin *et al.* 2005]

GvHD, graft-*versus*-host disease; aGvHD, acute GvHD; cGvHD, chronic GvHD; PB, peripheral blood; PT, post-transplant; Treg, regulatory T cells.

from activated effector T cells can be difficult based on this single marker.

FOXP3

The transcription factor, FOXP3, was found to be critical for Treg development and function in mouse models [Fontenot *et al*. 2003; Hori *et al*. 2003; Khattri *et al*. 2003], and hence provided a unique marker for Treg, although the intracellular localization of FOXP3 precludes its use in purifying Treg for functional studies. Activation of $CD4^{+}CD25^{-}$ T cells does not lead to FOXP3 expression in the mouse, unlike CD25 [Khattri *et al*. 2003]. In humans, mutation of the *FOXP3* gene causes a lymphoproliferative syndrome called IPEX (immune dysregulation, polyendocrinopathy, enterophy, and x-linked) [Sakaguchi *et al*. 2008]. However, FOXP3 expression in and of itself is inadequate to define human Treg, as naïve T cells without regulatory function can transiently upregulate FOXP3 upon activation [Gavin *et al*. 2006]. These findings have led to confusion on how to interpret results based on CD25 and FOXP3 expression in human Treg.

CD127 and CD45RA

CD127 (IL7 receptor alpha chain) and CD45RA have recently emerged as additional markers of Treg. In combination with high levels of CD25, low expression of CD127 is correlated with Treg that express FOXP3 [Liu *et al*. 2006; Seddiki *et al*. 2006]. However, conventional T cells can also reduce CD127 expression following activation *ex vivo*, limiting the ability to distinguish Treg from activated nonregulatory T cells [Mazzucchelli and Durum, 2007]. It is not clear whether this downregulation of CD127 occurs *in vivo*, particularly under inflammatory conditions such as GvHD. CD45RA and CD45RO are markers of maturity in human T cells and when used with CD25, define several subsets of Treg that share developmental origins but have distinct gene expression [Miyara *et al*. 2009]. $CD4CD25^{int}CD45RA+$ and $CD4CD25^{hi}CD45RO$ have low and high FOXP3 expression and are defined as resting and activated Treg, respectively. Both Treg subsets are suppressive *in vitro*, but the resting Treg are resistant to apoptosis and can proliferate in culture, while activated Treg have a more differentiated effector phenotype and are hyporesponsive to TCR stimulation. Additional markers, such as CD31, ICOS, and HLA-DR define additional cell populations within the resting and activated Treg subsets that appear to have differential suppressor function [Miyara *et al*. 2009; Ito *et al*. 2008; Baecher-Allan *et al*. 2006]. Thus, it is becoming clear that there is a heterogeneous population of human Treg, but whose function and localization *in vivo* remain unclear.

Natural Treg versus inducible Treg

Natural Treg (nTreg) are generated in the thymus, while 'inducible' or 'adaptive' Treg (iTreg) are produced in secondary lymphoid organs and peripheral tissues upon exposure to transforming growth factor beta (TGFβ) and retinoic acid [Coombes *et al*. 2007]. Other agents have been studied to convert naïve CD4 T cells into iTreg. These include hypomethylating agents (i.e. decitabine), antithymocyte globulin, and vasoactive intestinal peptides, with further details provided in Table 2. Both Treg subsets are mediators of peripheral tolerance and homeostasis in mouse models, however, the relative extent to which nTreg and iTreg contribute *in vivo* is not clear. This has been in part due to the absence of definitive markers that distinguish these two Treg subsets. Based on microarray studies in the mouse, two markers have been identified to be more specific for nTreg compared with iTreg. These include Helios [Thornton *et al*. 2010], an intracellular molecule, and Neuropilin 1 (Nrp1) [Bruder *et al*. 2004], a surface protein. While increased expression of Helios is observed for both mouse and human nTreg, early studies indicate that Nrp1 is only specific for mouse but not human nTreg. These surface markers provide tools to characterize the likely phenotypic differences and functional specialization between nTreg and iTreg *in vivo*.

In humans, the role of iTreg *in vivo* is not known. In vitro, human naïve CD4 T cells can be induced to express FOXP3 in the presence of TGFβ; however, these iTreg have reduced suppressor function *in vitro* and can secrete proinflammatory cytokines [Tran *et al*. 2007; Walker *et al*. 2005]. The addition of retinoic acid to the TGFβ condition appears to enhance stability and function of iTreg *in vitro* [Wang *et al*. 2009] however, whether this holds true in the *in vivo* setting, particularly under lymphopenic or inflammatory conditions, is not known. Also, these findings suggest that there may be other factors that are required to maintain stability of human iTreg.

Regulatory T-cell functional specialization

The mechanisms by which Treg suppress are complex, and include signaling via interleukin

Table 2. Strategies to induce or expand regulatory T cells and results in GvHD studies.

Strategy	Phenotype of resulting Treg	Outcome	PubMed ID [Reference]
HUMAN			
CONVERSION			
CD4+CD25- cells cultured with cell-based APCs and cilostamide	CD4+FOXP3+	9-fold increase in Treg:CD4+CD25- ratio; 100% survival at day 100 *versus* 0% survival at day 25 when mice given CD4+ effector cells	21593400 [Feng *et al.* 2011]
Patients received ECP for 6–12 months 17627234 [Biagi *et al.* 2007]	CD4+CD25+FOXP3+GITR+CD45RO+CD62L+	Secrete 4 × less IFNγ *versus* CD4+CD25- cells; Treg:CD4+ ratio increased 20%	
Naïve CD4+ and CD8+ cells cultured with vasoactive intestinal peptide matured DC	Two populations: (a) CD4+Tr1 cells (b) CD8+CD28-CTLA4+	Secrete negligible amounts of IL2, IL4, IL5 and 10 × more IL10, 4 × more TGFβ, 4 × less IFNγ secretion *versus* cells cultured with non-VIP DCs	16397128 [Gonzalez-Rey *et al.* 2006]
Leukapheresis products treated with anti-CD25	CD4+CD25^{hi}FOXP3+	49.5% conversion; 3 × greater suppression than CD25^{int} cells	16503495 [Hoffmann *et al.* 2006]
CD4+ cells cultured with anti-CD3, anti-CD52, and IL2	CD4+CD25+FOXP3+	20-fold expansion; 1.3 × greater suppression of CD4+ than non-CD52 Treg; 100% survival at day 21 in xenogeneic GvHD	16797237 [Watanabe *et al.* 2006]
CD4+CD25- cells cultured with CD40-activated B cells or iDCs	CD4^{hi}CD25	42.6% and 14.1% conversion of B cell and iDC group, respectively; 2.25 × greater suppression of CD4+CD25- cells than iDC group	20081875 [Zheng *et al.* 2010]
ECP for 6 months	CD4+CD25+FOXP3+CD127^{lo}	33% increase in TGFβ secretion *in vivo*	19773201 [Rao *et al.* 2009]
CD3+ cells cultured with anti-CD3, soluble anti-CD25, 5-azacytidine	CD4+CD25+FOXP3+CD127^{lo}	3.5-fold increase in FOXP3; 45% survival at day 50 *versus* 0% at day 35 in BM control of parallel mouse GvHD studies	19887673 [Sanchez-Abarca *et al.* 2010]
CD4+CD25- cultured with vasoactive intestinal peptide	CD4+CD25^{hi}FOXP3+	8% conversion; 3 × greater suppression than non-VIP Treg	19734220[Pozo *et al.* 2009]
CD4+CD25- cells with IL2 and rabbit antithymocyte globulin	CD4+CD25+FOXP3+	No suppression *in vitro*; FoxP3+ expression initially increased, but returned to baseline at day 10	19822903 [Broady *et al.* 2009]
EXPANSION			
PB CD4+CD25+ cells cultured with anti-CD3-loaded cell-based artificial APCs	CD4+CD25^{h}FOXP3+ CD127^{lo}	After 4 restimulations there was ~50 million fold yield, 55% purity; survival until day 80 *versus* survival until day 50 in PB cell group	21593401 [Hippen et al. 2011]
PB CD4+CD25+ cells cultured with anti-CD3, anti-CD28 beads	CD4+CD25+FOXP3+	285–1000-fold expansion; secrete no IFNγ; suppress 5 × more than CD4+CD25- cells	16699377 [Karakhanova *et al.* 2006]
CD4+CD25+ cells cultured with B cells, anti-CD28 beads, and IL2	CD4+CD25+FOXP3+	250-fold expansion; secrete negligible amounts of IL2 and IFNγ; suppressive *in vitro*	19684083 [Chen *et al.* 2009]
UCB units cultured with anti-CD3, anti-CD28 beads and IL2	CD4+CD25+CD127^{lo}	211 median expansion; median suppression of 86% *in vitro*; 43% incidence of aGvHD *versus* 61% in historical controls	20952687 [Brunstein *et al.* 2011]
CD4+CD25+ cultured with anti-CD3, anti-CD28, and IL2	CD4+CD25+FOXP3+CTLA-4CD62L+	100-fold expansion after 2 weeks; secrete 7 × less IFNγ than CD4+CD25- cells; 90% survival at day 30 in xenogeneic GvHD	19410243 [Cao *et al.* 2009]

(continued)

Table 2. Continued

Strategy	Phenotype of resulting Treg	Outcome	PubMed ID [Reference]
CD4+CD25+ cells cultured with CD4+CD8+ cells and gfp 120	CD4+CD25+CD45+	1.5× greater weight gain at day 40 than BM control in xenogeneic GvHD model	19439734 [Becker *et al.* 2009]
$CD4+CD25^{hi}CD127^{lo}$ cells from PB with anti-CD3, anti-CD28, and IL2	CD4+FOXP3+	No response in single case of aGvHD; reduction of immunosuppressive agents in single case of cGvHD	19559653 [Trzonkowski *et al.* 2009]
MOUSE			
CONVERSION			
CD4+CD25- cells with Decitabine or 5Aza, anti-CD3, anti-CD28, IL2	CD4+CD25+FoxP3+	1.6× greater suppression *versus* non-Dec Treg; 50% survival at day 100 compared *versus* 20% in control without 5-Azacitadine	20424188 [Choi *et al.* 2010]
$CD4+CD25+CD62L^{high}$ cells with anti-CD3/anti-CD28 beads, IL2 or splenocytes and IL2	$CD4+CD25+CD62L^{high}$	300- and 600-fold expansion for bead and splenocyte Treg, respectively; 100% survival at day 60 *versus* 30% in BM control	16394018 [Trenado *et al.* 2006]
CD4+CD25- cells with anti-CD3, anti-CD28, TGFβ and Ciglitazone	CD4+CD25+FoxP3+	80% conversion; 35% survival at day 50 *versus* 0% survival at day 20 in BM control	17371968 [Wohlfert *et al.* 2007]
Naïve CD4+ cells cultured with CD40-activated B cells	$CD4+CD25^{hi}$	31% conversion; 40× greater suppression of CD4+CD25- cells than CD4+CD25- cells; expression of Tbet, IFNγ, and CXCR3	21182084 [Zheng *et al.* 2011]
CD4+CD25- cells transfected with FoxP3-GFP vector	CD4+CD25+FoxP3+	56–68% conversion; 80% survival rate at day 100 *versus* 0% at day 40 in BM control	20018376 [Cao *et al.* 2010]
CD4+CD25- cells cultured with CD3epsilon+ or CD3epsilon- DCs	CD4+CD25+FoxP3+	12% and 17% conversion in CD3ε+ group and CD3ε-, respectively	18457820 [Taylor *et al.* 2008]
Spleenocytes cultured with antimurine thymocyte globulin	CD4+CD25+FoxP3-	4× greater expression of CD25 than splenocytes with rabbit IgG control; 3× greater survival rate at day 40	18025149 [Ruzek *et al.* 2008]
CD4+CD25- cells cultured with TGFβ, CD28 beads, and IL2	CD4+CD25+FoxP3+	35.9% and 5.3% conversion with and without CD28, respectively; 40% survival in CD28-deficient mice at day 80 *versus* 0% with control	21245484 [Semple *et al.* 2011]
CD4+CD25- with DCs and IFNγ	CD4+CD25+CD62L+FoxP3+	IFNγ group prevented graft rejection; DC/IFNγ group had 4× greater allograft acceptance than control	18724229 [Feng *et al.* 2008]
CD4+FoxP3- cells cultured with RA, IL2, and DCs	CD4+FoxP3+CD62L+	Revert into FOXP3-CD4+ T cells and did not protect from GvHD	19750478 [Koenecke *et al.* 2009]
EXPANSION			
CD4+CD25+ cells with artificial APCs expressing 4-1BBL or OX40L	$CD4+CD25^{hi}FoxP3+$	1200- and 1000-fold expansion for 41BBL and OX40L, respectively; 1.7× greater survival rate than bead-expanded Treg group	18645038 [Hippen *et al.* 2008]
CD4+CD25+ cells cultured with trichosanthin	$CD4+CD25^{hi}FoxP3+$	65–75-fold expansion; 60% survival at day 98 compared to 40% and 20% in natural and activated-Treg, respectively	20384869 [Wang *et al.* 2010]
Keratinocyte growth factor injection	CD4+FoxP3+	Treg:CD4+ ratio increased from 0.07 to 0.2 at day 5; decreased to 0.1 at day 20	18025186 [Bruinsma *et al.* 2007]

(continued)

Table 2. Continued

Strategy	Phenotype of resulting Treg	Outcome	PubMed ID [Reference]
CD4+CD25+ cells with BM-derived dendritic cells or spleen APCs	CD4+CD25+FoxP3+GITR+	2–5-fold expansion; 40% survival at day 50 *versus* 0% survival at day 20 in BM control	16473944 [Yamazaki *et al.* 2006]
Fms-like tryosine kinase 3 ligand expands DCs which expand CD4+CD25+ cells	FoxP3+	Treg:CD4+ ratio increased 2-fold; 5 × greater survival rate *versus* control	19211508 [Swee *et al.* 2009]
Superagonistic CD28-specific monoclonal antibody injection	CD4+CD25+FoxP3+	Treg:CD4+CD25+ ratio 0.9 in PB; 40% survival at day 60 *versus* 0% in BM control	20573297 [Kitazawa *et al.* 2010]
Injection of early-stage apoptotic spleen cells	CD4+CD25+CD62L+CTLA4+	2.5-fold increase in FoxP3; 10% survival at day 70 *versus* 0% at day 40 in BM control	15962005 [Kleinclauss *et al.* 2006]
Liposomal formulation of alpha-galactosylceramide injection	CD4+FoxP3+	10-fold expansion in spleen; significantly prolonged survival	21145405 [Duramad *et al.* 2011]
Anti-IFNγ injection	CD4+FoxP3+	2–4-fold decrease of FoxP3+ in spleen, liver, and skin	21263067 [Yi *et al.* 2011]

ECP, extracorporeal photophoresis; DC, dendritic cell; APC, antigen-presenting cell; PB, peripheral blood; GvHD, graft-*versus*-host disease; aGvHD, acute GvHD; cGvHD, chronic GvHD; Treg, regulatory T cells; iDC, immature dendritic cell; IL, interleukin; UCB, umbilical cord blood; TGF, transforming growth factor; IFN, interferon; BM, bone marrow.

10 (IL10), IL35, TGFβ, CD39, CTLA4, LAG3, Granzyme A and B, and perforin, among others. The details of these mechanisms are beyond the scope of this review, and were summarized recently [Vignali *et al.* 2008]. Most of the mechanistic studies were derived from mouse models. Our current understanding of how human Treg function remains limited to *in vitro* studies, which do not account for the importance of migration and localization in the development, differentiation, and function of Treg *in vivo* [Zhang *et al.* 2009]. Thus, questions remain on where adoptively transferred human Treg get primed, how they get there, and where they function. Some insights have been provided by mouse models of allogeneic tissue grafts [Zhang *et al.* 2009]. In these studies, nTreg enter the inflamed tissue before reaching the draining lymph node (LN). In contrast, iTreg first enter the LN before migrating to the site of inflammation. These observations suggest that nTreg and iTreg have different homing receptors at transfer that affect their site of priming and activation *in vivo.*

Recent studies indicate that there is a heterogeneous population of Treg with functional specialization [Campbell and Koch, 2011]. In particular, there are subsets of human Treg with distinct expression of transcription factors, cytokines, and chemokine receptors that parallel those expressed in subsets of helper T cells (Th). How and from whence these Treg develop and acquire similar phenotypic features of their Th counterpart requires further investigation. Preliminary studies suggest that nTreg responds to cytokines that are specific to the Th subset, for example, interferon gamma (IFNγ), and upregulate Tbet, a transcription factor normally expressed in Th1 cells, which promotes acquisition of homing receptors that allow accumulation of Treg at sites of Th1 mediated immunity [Koch *et al.* 2009]. These findings are relevant as we continue to define subsets of Treg that are most effective for different diseases.

As noted previously, nTreg and iTreg are developmentally different. Therefore, is not clear whether iTreg and nTreg share mechanisms of suppression. Because iTreg are generated by multiple pathways, some not dependent on FOXP3 expression, there are likely mechanisms of suppression that are unique to iTreg. One example is IL35, a cytokine consisting of IL27β and IL12α subunits, that are increased on Treg, but not conventional T cells. In both mouse and humans,

IL35 induces iTreg, which continue to generate IL35 to sustain the iTreg population and function [Collison *et al.* 2010]. IL35-Treg do not express FOXP3 or IL10, but function *in vivo* with the same or higher suppressive capacity than nTreg. Other iTreg subsets that express FOXP3 are not as potent suppressor *in vivo* compared with nTreg and this is likely related to the functional dependence of these iTreg on FOXP3 expression, which is transient and unstable *in vivo* as described below. Under homeostatic conditions, IL35-Treg are not detected; they require very strong inflammatory conditions for induction and expansion [Collison *et al.* 2010]. Potential differences in nTreg and iTreg suppression suggest that they play nonoverlapping roles *in vivo* and therefore may have differential impact in different model systems.

The stability of the Treg phenotype and function *in vivo* remains in question [Sakaguchi, 2010]. Understanding this aspect of Treg biology is critical as we consider the subset(s) of Treg that can be used in adoptive transfer studies and in the clinical settings. In murine studies, nTreg appears to have a stable suppressor phenotype and function, even under inflammatory conditions [Rubtsov *et al.* 2010]. In contrast, both mouse and human studies indicate that FOXP3 expression in iTreg is more transient, and hence the suppressor function is inferior *in vivo.* This is particularly true under inflammatory or lymphopenic conditions, which are generally the setting under which iTreg would be adoptively transferred [Koenecke *et al.* 2009; Zhou *et al.* 2009; Tran *et al.* 2007; Walker *et al.* 2005]. Using FOXP3 lineage-tracer mice, it was shown that iTreg are less stable and become 'ex-Treg' that generate IFNγ and can induce diabetes in NOD mice [Zhou *et al.* 2009]. The plasticity of the iTreg must therefore be taken into account as we consider their use *in vivo* and in the clinical setting. iTreg subsets that are FOXP3 independent, however, can remain suppressive under inflammatory conditions such as the IL35 iTreg aforementioned.

GvHD, graft-versus-tumor, and Treg

Mouse studies

Early studies in various murine models of bone marrow transplantation indicate that adoptively transferred Treg can prevent and treat GvHD [Nguyen *et al.* 2007; Edinger *et al.* 2003; Jones *et al.* 2003; Cohen *et al.* 2002; Taylor *et al.* 2002]. The mechanisms by which Treg suppress GvHD is not entirely clear, but localization of Treg into secondary lymphoid organs [Taylor *et al.* 2004; Edinger *et al.* 2003] and peripheral tissues [Wysocki *et al.* 2005] are critical for their function. Bioluminescence imaging studies have provided insights into the kinetics of Treg in GvHD [Nguyen *et al.* 2007]. These studies demonstrate that Treg, purified as $CD4^{+}CD25^{hi}$ cells, colocalize with conventional effector T cells (Tcon) in secondary lymphoid organs initially, where they expand robustly and then migrate into peripheral tissues such as the gut and skin. Their colocalization also led to the suppression of Tcon proliferation in both lymphoid and peripheral tissues. After approximately 7–10 days following transfer, there is a significant reduction in Treg numbers, which may due to a decreased inflammatory milieu. By bioluminescence imaging, these labeled Treg, although reduced, are still detectable 3 months following transfer and are found diffusely in peripheral tissues. These findings provided a framework for the design and development of some recent clinical trials for adoptive Treg transfer.

Correlative human studies

Many correlative studies have been published to establish an association between the level of Treg in various tissues and the incidence and/or severity of GvHD in patients. As detailed in Table 1, the results are conflicting and may be due to several reasons, particularly variability in the Treg phenotype, the time point at which they are analyzed, and the tissue from which they are extracted for analysis. Predominantly, most studies report on peripheral blood (PB) Treg number and percentages; however, the relevance of PB Treg and how they reflect Treg number and function in the tissue are not known, particularly in light of functional specialization and localization of different Treg subsets. Another caveat is that the kinetics of Treg and their role in causing or preventing disease *in vivo* cannot be captured by a single or even a few time points following transplantation. Therefore, it would difficult to determine whether low numbers of Treg in the gut of patients with GvHD can explain cause or effect based on these studies. Furthermore, strategies to purify or characterize 'Treg' are different between experiments or studies, complicating interpretation of the findings across studies. Nevertheless, the majority of the studies indicate an inverse correlation between Treg number or

Treg:Tcon ratio and the incidence and severity of GvHD.

Phenotype and source of Treg for adoptive therapy

Most preclinical studies in transplantation transfer freshly isolated Treg. However, because Treg comprise a small fraction of CD4 T cells, a concern is an insufficient number of Treg that can be isolated for adoptive transfer in the clinical setting. To address this potential issue, based on preclinical data [Cohen *et al*. 2002; Taylor *et al*. 2002] current ongoing efforts are to expand nTreg under activating conditions with CD3/CD28 stimulation and IL2, or to induce naïve $CD4^+CD25^-$ T cells under skewing conditions with high-dose TGFβ and/or retinoic acid that promote conversion into iTreg. Other strategies for expansion and conversion have recently been published and are detailed in Table 2. While expansion and conversion strategies generate significant numbers of Treg, there are issues of purity and *in vivo* functional stability for *ex vivo* expanded nTreg and iTreg, respectively.

As noted previously, multiple studies have demonstrated plasticity and lack of stability of the phenotype and function of iTreg in autoimmune models. Furthermore, in a recent study, iTreg generated *ex vivo* under TGFβ conditions reverted to a nonregulatory T-cell phenotype *in vivo* and failed to prevent GvHD in a murine model [Koenecke *et al*. 2009]. Thus, TGFβ-induced Treg do not appear to be a reliable source of suppressor cells for transfer under inflammatory and lymphopenic settings. However, as detailed in Table 2, there are a multitude of strategies that may induce a more stable phenotype but these strategies require further studies to confirm. For the induction of human Treg, several groups have demonstrated that a course of extracorporeal photopheresis could convert naïve T cells into iTreg *in vivo* [Rao *et al*. 2009; Biagi *et al*. 2007]. Other investigators showed stable iTreg induction with hypomethylating agents [Sanchez-Abarca *et al*. 2010], a promising strategy for translation in the clinical setting but requires further testing.

For *ex vivo* expanded nTreg, contamination with effector T cells in the culture system remains the major concern. In part, this is due to the imprecise selection of Treg based on CD25, since activated T cells also upregulate this marker. However, with additional markers such as CD127 and CD45RA as detailed previously, it may now be possible to increase the purity of sorted and expanded Treg for adoptive transfer studies [Hippen *et al*. 2011].

A second concern regarding expanded nTreg is the impact culturing may have on their homing and chemokine receptor expression, which could interfere with their effective entry into target tissues. In murine models of GvHD, expanded nTreg can suppress GvHD efficiently, albeit it appears that the major suppressive activity is due to the $CD62L^{hi}$ Treg subset [Taylor *et al*. 2004]. Although one may postulate homing receptor expression would change with activation *in vitro*, a recent study and our own experience showed that activation of nTreg *in vitro* under CD3/CD28 costimulation does not significantly impact the chemokine receptor or selectin expression: CD62L expression remained high and there was no affect on the expression levels of CCR5, CCR6, CCR8, CLA, or CD103 [Cao *et al*. 2009]. Further studies are needed to confirm the *in vivo* homing capacity of expanded nTreg compared with freshly isolated Treg. Results may help determine the appropriate cell phenotype and dose for testing in the clinical trial setting since the ability of Treg to suppress *in vivo* depends on their ability to migrate to the right target tissue.

Until concerns of purity and stability of iTreg and expanded nTreg are addressed, freshly isolated nTreg are the most appropriate source for clinical trial. The optimal dose of Treg in the clinical setting is not known, but likely depends on the timing of adoptive transfer, the number effector T cells in the graft, and the tumor burden of the patient. While preclinical studies indicate that a 1:1 nTreg:Tcon ratio is effective in suppressing GvHD without abrogating the graft-*versus*-tumor response [Edinger *et al*. 2003; Cohen *et al*. 2002], it is not clear that this strategy would lead to similar outcomes in patients. Ongoing clinical trials of Treg immunotherapy will help establish this dose parameter.

Most preclinical studies and all clinical trials to date transfer polyclonal Treg into transplant recipients. Antigen-specific Treg (Ag-Treg) have been evaluated in autoimmune disease models to reduce nonspecific suppression of host immunity [Filippi *et al*. 2005]. For GvHD however, it is not currently possible to generate Ag-Treg given the lack of known immunodominant antigens and the multi-tissue expression of alloantigens and

minor histocompatibility antigens that contribute \to the pathologic immune response. In models of GvHD, polyclonal Treg do not appear to compromise the GvT effect [Edinger *et al*. 2003; Cohen *et al*. 2002]. However, these studies were performed under contrived experimental conditions, with specific cell dose ratios and transplantable leukemia which may not be translatable to the clinical setting. Whether polyclonal Treg suppress tumor and microbial immunity following hematopoietic-cell transplantation (HCT) in the clinical setting remains unknown, and thus careful monitoring for relapse and infections are critical in trials incorporating Treg immunotherapy.

Clinical trials

Recently, two clinical trials reported on the outcomes of infusing Treg in patients undergoing haploidentical [Di Ianni *et al*. 2011] and double-cord HCT [Brunstein *et al*. 2011]. In the former study performed in Italy, 28 patients with hematological malignancies, primarily acute myeloid leukemia (AML), underwent myeloablative conditioning with total body irradiation (TBI), thiotepa, fludarabine, and cyclophosphamide followed by an infusion of freshly isolated $CD4^+CD25^+$ Treg from a haploidentical donor. After 4 days, the patient received infusions of CD34-selected progenitor cells and conventional CD3 T cells (Tcon) separately. This was a dose escalation phase I trial, with dose ratios of Treg:Tcon ranging from 4:1 to 2:1, with a maximum of 4×10^6/kg Treg given. The patients did not receive any post-transplant GvHD prophylaxis. With a median follow up of approximately 12 months, the authors report a reduction in GvHD compared with historical controls; no patients developed GvHD when 1×10^6/kg or less Tcon were given. Furthermore, donor immune reconstitution was timely, with earlier recovery of T cells specific to various opportunistic pathogens, including Aspergillus, cytomegalovirus (CMV), and adenovirus, congruent with preclinical data [Nguyen *et al*. 2008]. There were fewer CMV reactivations and no CMV-related mortality compared with their prior experience [Perruccio *et al*. 2005]. Overall, at a median follow up of 12 months, survival was 46%, with the majority of deaths due to infection, and the treatment related mortality (TRM) was 50%, which is higher than the reported 36.5% in their prior haploidentical HCT study [Aversa *et al*. 2005]. Leukemia relapse was not increased; however, longer follow up is required to determine the impact of Treg on the Graft versus leukemia (GvL) effect.

In the second clinical trial, Brunstein and colleagues reported results on the infusion of *ex vivo* expanded/activated umbilical-cord blood (UCB)-derived Treg [Brunstein *et al*. 2011]. A total of 23 patients with various leukemias underwent nonmyeloablative conditioning with low-dose TBI, cyclophosphamide, and fludarabine, followed by infusion of two HLA 4-6/6 UCB graft. From a third-party UCB, Treg were positively selected for CD25, expanded in culture with anti-CD3/anti-CD28 monoclonal antibody and recombinant IL2 over a 14-day period, and infused into the patient on day +1 of HCT. For 14 patients, a second infusion of Treg was given at day +15. This was a dose-escalation trial, with dose levels of 1, 3, 10, or 30×10^5 Treg/kg. GvHD prophylaxis consisted of mycophenolate mofetil (MMF) and cyclosporine in the first cohort, and MMF and sirolimus in a subsequent cohort based on reports that CsA may interfere with Treg function and survival [Coenen *et al*. 2006; Zeiser *et al*. 2006]. At a median follow up of approximately 1 year, Treg recipients had similar disease-free survival, long-term donor engraftment, and nonrelapse mortality compared with historical controls [Brunstein *et al*. 2007]. As with the trial by Di Ianni and colleagues, longer follow up is required to determine the impact of Treg on the GvL effect. While the incidence of grades II–IV acute GvHD was lowered in Treg-treated patients than historical controls, no difference was noted for grades III–IV acute GvHD. The role of Treg in chronic GvHD in this trial is not clear at the time of report given the short follow up.

Results from these two clinical trials indicate the feasibility of Treg infusions. However, the results also raise several questions of safety and efficacy. First, what are the reasons why relapse is not increased in Treg recipients? Overall, follow up in both studies is too short to conclude firmly. However, the infusion of Treg allowed the add-back of Tcon which may exert a GvL effect in the haploidentical HCT trial. In the UCB HCT, the authors show short-term survival of Treg of approximately 2 weeks following infusion; thus, perhaps the threshold of effector cells that survive are sufficient to mediate GvL. However, studies of the *in vivo* kinetics of Treg were limited to circulating Treg in the blood, and not in lymphoid or peripheral tissues where Treg reside

and function. Thus, further investigation is needed to determine why Treg, particularly at the higher doses, do not impact tumor immunity in these two trials.

Second, in the haploidentical HCT trial, TRM was higher compared to historical controls, and are primarily due to opportunistic infections, even in the absence of standard immunosuppressive agents. Recent studies in viral infection models indicate that Treg can impair CD8 T-cell cytolytic activity, thus promoting chronic infection [Dietze *et al*. 2011]. It's not clear whether Treg has similar effects on the mature donor Tcon which are important for early microbial immunity after transplant. However, differences in the preparative regimen and the patient population may also account for the increased TRM in this trial.

Third, in the UCB HCT clinical trial, the expanded Treg did not prevent high-grade acute GvHD compared with historical controls. This may be due to a variety of reasons, including timing of Treg infusion, differences in potency of fresh *versus* expanded Treg, the number and ratio of Treg relative to Tcon, and the purity of the Treg infused. In the haploidentical HCT trial, Treg were infused 4 days prior to Tcon, which likely increased the Treg:Tcon ratio based on Treg expansion during this lapsed time as shown in preclinical models [Nguyen *et al*. 2007]. In contrast, in the UCB HCT trial, Treg was infused 1 day after Tcon. In this situation, the earlier Tcon infusion may lead to early Tcon expansion, hence reducing the Treg:Tcon ratio. The timing of Treg therefore may have an impact on the final ratio of Treg:Tcon *in vivo* and affect GvHD outcomes. Furthermore, in the UBC HCT trial, the median proportion of $CD4^+CD25^+$ cells was 65% following CD25 selection, and the median proportion of $CD4^+CD127^-FOXP3^+$ was 64% after culture. The 'Treg' product therefore likely consists of a significant proportion of Tcon which may offset the suppressive effects of Treg and may lead to outcomes that are more difficult to interpret. Improving the purity of the Treg population for adoptive transfer, either through flow-cytometric sorting or cultures under rapamycin conditions [Zeiser *et al*. 2008; Coenen *et al*. 2007; Battaglia *et al*. 2005] may address these concerns. Furthermore, performing a larger and randomized trial with long-term follow up will better at demonstrating the efficacy of Treg infusion.

Double UCB HCT typically leads to engraftment of a single unit at day 100 [Barker *et al*. 2005], suggesting rejection of the second unit by residual host cells, donor cells from the engrafted cord, or both. The biology of this engraftment dominance of a single unit is not well understood. In the UBC HCT trial by Brunstein and colleagues, the transferred Treg are derived from a third UCB unit. Analysis of the kinetics for the administered Treg indicates a reduction in both their absolute number and their percentage of total CD4 cells by day 14 post-HCT. While this decrease in Treg may be consistent with a reduction in inflammation further out from HCT, it does raise the question of whether third-party Treg are at risk for rejection by host residual cells or cells from the other two UCB units. Some preclinical findings suggest that third-party Treg may not be protective of engraftment [Gaidot *et al*. 2011; Joffre et al. 2004]; however, other studies in both mouse and xenogeneic models of GvHD show promise of third-party Treg as an effective 'off-the-shelf' therapy [Hippen *et al*. 2011; Steiner *et al*. 2006].

Ongoing trials are accessing the role of Treg in GvHD and other diseases, including diabetes and kidney transplant rejection. For diabetic patients, trials include adoptive transfer of polyclonal expanded autologous Treg ($CD4^+CD25^+CD127^{lo}$) as a single infusion, or administration of low-dose IL2 to induce Treg *in vivo*. In the allogeneic HCT setting, studies include a phase 2 trial to test *in vivo* induction of Treg with low-dose IL2 as GvHD prophylaxis, and a phase 1 trial that transfers sorted freshly isolated polyclonal nTreg ($CD4^+CD25^+CD127^{lo}$) into haploidentical graft recipients to allow higher doses of Tcon to be given without inducing GvHD.

Treg in evolving or established GvHD

Studies to date have primarily evaluated the role of Treg, given prophylactically, in reducing the incidence and severity of GvHD. The impact of Treg on established or evolving GvHD has been less studied [Nguyen *et al*. 2007; Jones *et al*. 2003]. When transferred into mice with established GvHD, Treg were less effective and while recipients of Treg had improved survival, end organ damage caused by GvHD was not reversed. In a case report, *ex vivo* expanded

$CD4^+CD25^+CD127^-$ Treg were infused into two patients with steroid-refractory GvHD [Trzonkowski *et al*. 2009]. The first patient had grade IV acute GvHD and did not respond to three weekly infusions of 3×10^6 Treg/kg. The second patient with pulmonary chronic GvHD received a single dose of 1×10^5 Treg/kg, which lead to a reduction in symptoms and allowed tapering of multiple immunosuppressive agents. However, the long-term outcome of this patient is not known. These findings indicate that Treg immunotherapy may be optimal under prophylactic conditions.

Summary

Preclinical data indicate that Treg are a potent suppressor of GvHD that do not abrogate tumor and microbial immunity under specific dose ratios with Tcon. However, it remains unclear whether these outcomes would hold in the clinical setting, given our limited understanding of how human Treg function *in vivo* and challenges in achieving the desired cell dose ratios and purity. Moreover, unlike the mouse model, significant variability exists in our patients, including their genetics, disease, and the transplant and immunosuppressive regimens with which they are treated, all of which may have an impact on their response to Treg immunotherapy and other transplant outcomes. Two clinical trials offer preliminary observations which demonstrate the feasibility and safety of Treg infusion. However, long-term outcomes on efficacy and bystander effects on tumor and microbial immunity must be further addressed in randomized and larger trials. With emerging data on heterogeneous populations of human Treg with functional specialization, and as our ability to purify Treg advances with new markers, the benefit of Treg immunotherapy may rely on transferring specific subsets of Treg for different diseases, and even for different organs affected as is the case for GvHD. However, as with many cellular therapies, the labor- and cost-intensive process of generating the product may preclude their wide application to less-specialized centers and, as such, efforts to streamline this step must parallel our studies in understanding the biology and clinical use of Treg.

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