Immunity
Review

Human T Regulatory Cell Therapy: Take a Billion or So and Call Me in the Morning

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Immune system regulation is of paramount importance to host survival. In settings of autoimmunity and alloimmunity, control is lost, resulting in injury to vital organs and tissues. Naturally occurring, thymic-derived T regulatory (Treg) cells that express CD4, CD25, and the forkhead box protein 3 (FoxP3) are potent suppressors of these adverse immune responses. Preclinical studies have shown that either freshly isolated or ex vivo expanded Treg cells can prevent both local and systemic organ and tissue destruction. Although promising, human Treg cell infusion therapy has heretofore been difficult to implement in the clinic, and relatively few clinical trials have been initiated. This review will focus on the preclinical models that provide the rationale for current trials and it will address both the challenges and opportunities in human Treg cell therapy.

Introduction

The immune system serves as a barrier against foreign pathogens and abnormal cell growth. To prevent excessive or indiscriminate immune responses that might compromise survival of the organism, several nonredundant regulatory mechanisms exist to maintain this delicate balance. The focus of this review stems from the seminal work of Sakaguchi et al. that report the transfer of $CD25^+$-depleted $CD4^+$ T cells into nude mice resulted in autoimmune disease (Sakaguchi et al., 1995). The disease course was reversed by adding $CD4^+CD25^+$ T cells (now termed regulatory T [Treg] cells). The importance of Treg cells in immune regulation has been well established in mice and humans. Although the therapeutic potential of Treg cells was envisioned decades ago (Gershon, 1975), clinical implementation of their potent immune regulatory activity has proven challenging. Like conventional T (Tconv) cells, Treg cells need T cell receptor (TCR) triggering and costimulation to become fully active. Although some costimulatory pathways may differentially regulate Treg cells and Tconv cells (Riley and June, 2005), no single pathway is known to regulate one cell type exclusively. Thus, in vivo administration of agents that augment Treg cell activity may also augment Tconv cell activity. This point was most dramatically illustrated in a phase I clinical trial testing TGN1412, a superagonistic CD28 antibody (Ab) (Suntharalingam et al., 2006). Preclinical data in animal models demonstrated that this Ab preferentially activated Treg cells, and it was thus postulated that TGN1412 could restore tolerance and enable transplants (Beyersdorf et al., 2005; Lin and Hunig, 2003). Unfortunately, when seemingly low Ab doses were delivered to six healthy adults, a massive cytokine storm ensued, and only prompt and intensive medical intervention prevented deaths (Suntharalingam et al., 2006). The precise reasons why these adverse events were not observed in rodent and nonhuman primate models are not known, but this trial underscores the danger of injecting agents designed to modulate T cell activity in vivo without being able to selectively target specific T cell subsets.

Adoptive Treg cell therapy is an attractive alternative to harness the immune suppressive activity of Treg cells (June and Blazar, 2006). In this approach, Treg cells are isolated from a patient, enriched, expanded ex vivo, and reinfused. This approach is advantageous because the expanded product can be analyzed phenotypically and functionally prior to infusion, providing another level of safety. Furthermore, cell dosage can be tightly controlled. Despite these advantages, adoptive Treg cell therapy is just now being tested in the clinic. This review will evaluate the rationale for the clinical use of ex vivo expanded Treg cells and it will emphasize the difficulties, as well as the opportunities, encountered in transitioning from the bench to the bedside.

Overview of Autoimmune and Alloimmune Immune Regulation

Autoimmunity can be defined as the loss of self-tolerance. It can arise from genetic lesions, molecular mimicry, or environmental stress that overrides the immune system's safeguards against self-attack (Christen and Herrath, 2004). Once these protective measures are overcome, the self immune response shares many features with the non-self immune response, including MHC-restricted antigen specificity and immune memory generation.

Autoimmune disease can be directly caused by Treg cell dysfunction. The clearest example is immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, in which loss-of-function mutations in the FoxP3 gene lead to severe autoimmunity. However, there are many instances in which the role of Treg cells in autoimmunity is unclear. For example, CTLA-4 polymorphisms are associated with autoimmunity (Scalapino and Daikh, 2008). This association was initially attributed to a lowered threshold for Tconv cell activation consequent to diminished Treg cell CTLA-4 expression, resulting in greater activation of self-reactive T cells (Egen et al., 2002). However, recent studies have indicated that CTLA-4 is also

important for Treg cell function (Friedline et al., 2009; Flores-Borja et al., 2008; Wing et al., 2008). Obviously, these scenarios are not mutually exclusive. Nonetheless, the rationale to adoptively transfer autologous Treg cells to treat autoimmune disease associated with the loss of CTLA-4 activity would be stronger if Treg cell function is reasonably preserved and autoimmunity is due to higher numbers of autoreactive T cells. Here, the addition of functional Treg cells may reset the proper balance between tolerance and immunity. On the other hand, if CTLA-4 activity is required for Treg cell effector functions, then the rationale of infusing additional non-fully functional Treg cells is not so clear.

The precise contribution of reduced Treg cell numbers and reduced Treg cell activity to autoimmunity is an important unresolved issue. Although reduced numbers of Treg cells have been observed in type I diabetes (T1D) patients (Kukreja et al., 2002), the majority of reports indicate that T1D patients and age-matched healthy controls have similar numbers of Treg cells (Putnam et al., 2008; Brusko et al., 2007; Lindley et al., 2005). Although freshly isolated T1D patient Treg cells have been reported to be less effective suppressors than Treg cells from healthy control donors, it was not clear from these studies whether the Treg cells were less effective or whether the responder Tconv cells were more resistant to suppression (Schneider et al., 2008; Lindley et al., 2005). Importantly, Treg cells from a T1D patient, grown ex vivo with a suboptimal T cell expansion system incorporating rapamycin (a pharmacological inhibitor of Tconv cell responses), suppressed allogeneic Tconv cells as potently as similarly cultured Treg cells from healthy controls. These data suggested that functional deficits in Treg cells from T1D patients were corrected during the expansion process (Battaglia et al., 2006). This suggestion is supported by a recent study indicating that Treg cells from T1D patients are functionally similar to those from healthy controls and that autologous responder Tconv cells from T1D patients are more difficult to suppress in vitro (Putnam et al., 2008). Thus, greater insights into the biological and molecular defects underlying given autoimmune diseases may guide the decision to pursue immunotherapy with adoptively transferred Treg cells.

In contrast to the autoimmune response to self-antigens, alloimmunity involves the recognition of foreign gene products encoded by polymorphic MHC and minor histocompatibility antigen loci. Although both the innate and adaptive immune systems contribute to an alloresponse, the dominant effects are mediated by allogeneic $CD4^+$ and/or $CD8^+$ Tconv cells, which can directly recognize foreign MHC molecules and associated peptides expressed on allogeneic antigen-presenting cells (APCs) or tissue cells, or indirectly recognize foreign peptides presented by syngeneic APCs (Rosenberg and Singer, 1992). After solid organ transplantation, alloreactivity is fueled by inflammation and cell injury that occurs locally and is limited in severity and duration. In contrast to hematopoietic stem cell transplantation (HSCT), central tolerance to foreign antigens present on transplanted solid organ grafts fails to occur. Therefore, long-term solid organ graft acceptance requires either continuous immune-suppressive drugs or the acquisition of peripheral regulatory mechanisms.

After myeloablative chemoradiotherapy conditioning for HSCT, systemic inflammation is severe and often overwhelms immune regulatory mechanisms, despite the routine use of multi-agent immunosuppressive drug regimens designed to dampen alloreactivity. Despite the more aggressive early post-HSCT inflammatory response and the purposeful infusion of donor T cells into a lymphopenic environment that maximally supports their expansion, HSCT actually fosters the development of tolerance as host APCs are replaced with donor APCs and T cells. Thus, the risk period for adverse donor antihost alloresponses that culminate in the multiorgan system disorder known as graft-versus-host disease (GVHD) typically is highest in the first 1–3 months after HSCT until central tolerance occurs. Therefore, the critical time of need for intense immune regulation for HSCT recipients can be precisely timed and effective immune suppression or tolerance induction during this relatively short window may provide life-long protection against adverse alloresponses without the need for immunosuppressive drugs. For these reasons, and because Treg cells can be isolated from healthy HSCT donors, human Treg cell trials in HSCT recipients have preceded other indications.

Preclinical Data in Support for Treg Cell Infusional Therapy in Autoimmunity

T1D results from the failure to control islet-specific Tconv cells. The role of Treg cells in the establishment and progression of T1D has been intensely studied. Nonobese diabetic (NOD) mice spontaneously acquire T1D and they have been the workhorse for murine studies of Treg cells and T1D progression (Anderson and Bluestone, 2005). Susceptibility to diabetes correlates with the loss of FoxP3-expressing Treg cells (Baecher-Allan and Hafler, 2006). T1D can be prevented by adoptive transfer of either freshly isolated polyclonal or antigen-specific Treg cells (Szanya et al., 2002; Tarbell et al., 2007; Tang et al., 2004). This argues strongly that Treg cells play a crucial role in the pathogenesis, and potentially the treatment, of T1D. In NOD mice lacking Treg cells, no differences in either the initial activation of Tconv cells in draining lymph nodes or the rate of Tconv cell islet infiltration were observed. However, accelerated islet destruction occurred in these mice (Chen et al., 2005), suggesting that adoptive transfer of functional Treg cells will be of benefit to patients with prediabetic lesions. Notably, 80% of IPEX patients develop T1D within a year after birth (Sakaguchi et al., 2006).

Although adoptive Treg cell therapy has been primarily focused on T1D and HSCT, there are numerous other diseases in which Treg cell therapy is worthy of consideration. In the aforementioned study by Sakaguchi and colleagues, mice depleted of Treg cells developed dsDNA Abs reminiscent of systemic lupus erythematosus (SLE) (Sakaguchi et al., 1995). Others have noticed that SLE patients have fewer Treg cells than healthy individuals, that such Treg cells are more susceptible to Fas-mediated apoptosis, and that Treg cells from patients with active disease have impaired activity (Crispin et al., 2003; Miyara et al., 2005). Notably, ex vivo expansion of Treg cells from SLE patients corrected the Treg cell functional defect (Valencia et al., 2007), suggesting that ex vivo expanded, autologous Treg cells might have a beneficial effect in SLE patients. Similarly, patients with Sjögren's syndrome have reduced numbers of Treg cells in their peripheral blood. Furthermore, SLE patients' salivary glands, a common autoimmune target in this population, contain markedly reduced numbers of Treg cells (Li et al., 2007). In Sjögren's syndrome patients, Treg cells functioned

equivalently on a per-cell basis as compared to age-matched healthy controls, suggesting that Treg cell infusion has therapeutic potential in this disease setting (Li et al., 2007).

In other autoimmune diseases, such as multiple sclerosis (MS), the rationale for Treg cell therapy is less clear. Results from murine models of MS (experimental autoimmune encephalomyelitis, EAE) question the therapeutic benefit of adoptive Treg cell transfer. If polyclonal or antigen-specific Treg cells are infused prior to acquisition of disease, EAE can be prevented. However, if the Treg cells are infused after disease initiation, their therapeutic value is considerably diminished (Olivares-Villagomez et al., 1998). Examination of the ability of antigen-specific Treg cells to suppress Tconv cells isolated from the CNS of animals with active EAE revealed that Tconv cells were highly resistant to the antiproliferative effects of antigen-specific Treg cells, perhaps resulting from the fact that these Tconv cells secrete high amounts of the cytokines IL-6 and TGF-β (Korn et al., 2007). There is evidence that Treg cells function poorly in inflammatory environments (Lewis et al., 2008). Because individuals with active autoimmune disease like MS are likely to have inflammatory environment at or near the target organ, pretreatment with anti-inflammatory agents such as alpha 1-antitrypsin (Koulmanda et al., 2008; Lewis et al., 2008) prior to infusion of ex vivo expanded Treg cells may be of benefit. Additionally, several studies have demonstrated that Treg cells isolated from MS patients have diminished suppressive activity (Haas et al., 2005; Viglietta et al., 2004). If this is so, and these defects are not reversed by ex vivo culture, it is questionable whether the infusion of additional, presumably defective, Treg cells will provide therapeutic benefit. Preclinical studies to determine whether autologous Treg cells derived from progenitor cells such as gene-corrected, patient-specific induced pluripotent stem cells derived from MS patients might circumvent these defects are now possible but have not yet been performed.

Preclinical Data in Support for Treg Cell Infusional Therapy in Alloimmunity

In cyclosporin-treated rats with long-term cardiac allograft survival, the adoptive transfer of $CD4^+CD25^+$ T cells resulted in tolerance (Hall et al., 1990). The demonstration that Treg cells from naive mice prevented rejection of allogeneic skin grafts in nude mice given $CD25^-$ Tconv cells (Sakaguchi et al., 1995) further set the stage for the application of Treg cells to solid organ transplant settings. In vitro studies in which positive costimulatory pathway blockade inhibited alloantigen-specific responsiveness demonstrated that Treg cells were essential in the tolerance induction process as assessed in vitro and in vivo in a GVHD model (Taylor et al., 2001). In vivo, tolerance induced by the combined administration of donor-specific transfusions and costimulatory pathway blockade was dependent upon Treg cells present in the transfusion product (Jarvinen et al., 2003). A series of preclinical rodent studies demonstrated that Treg cells present in the recipient at the time of skin or cardiac allografting were critical to tolerance induction and maintenance in vivo (reviewed in Wood and Sakaguchi, 2003). Moreover, the adoptive transfer of $CD4^+CD25^+$ or $CD4^+CD45RB^{lo}$ Treg cells suppressed pancreatic islet allograft rejection (Sanchez-Fueyo et al., 2002; Davies et al., 1999).

In murine models, depletion of $CD25^+$ T cells from the donor allograft accelerated both acute and chronic rejection (Anderson et al., 2004; Cohen et al., 2002; Hoffmann et al., 2002; Taylor et al., 2002). Conversely, the infusion of freshly isolated or ex vivo expanded donor Treg cells was highly effective in preventing acute or chronic GVHD (Taylor et al., 2002; Zhao et al., 2008). Further, in vivo activated donor or host Treg cells were able to ameliorate ongoing chronic GVHD (Anderson et al., 2004; Zhao et al., 2008). In sublethally irradiated recipients of T cell-depleted allogeneic bone marrow, host antidonor alloreactive Tconv cells could reject donor bone marrow, which was preventable by donor Treg cell infusion (Joffre et al., 2004; Hanash and Levy, 2005; Taylor et al., 2004). Donor Treg cell infusion also sped immune recovery and prevented GVHD-induced thymic involution (Trenado et al., 2003). Given the striking results in rodent GVHD and bone marrow graft rejection models, ready availability of donor Treg cells, known and transient risk period for adverse consequences from alloreactive T cells, and high degree of morbidity and mortality associated with HSCT in patients, it is not surprising that GVHD prevention has emerged as the first clinical application for human Treg cells.

Challenges and Clinical Applications of Human Treg Cell Large-Scale Manufacturing

Human Treg cells have no unique cell surface markers. Translating Treg cells into the clinic has been hindered by the relatively high frequency of activated Tconv cells present in the $CD4^+CD25^+$ fraction and the limited availability of good manufacturing practice (GMP)-compatible procedures for removing these contaminants. Furthermore, the relatively low frequency of Treg cells in human peripheral blood, combined with data from mouse models indicating that large doses of polyclonally activated Treg cells are required to suppress GVHD, indicate that for most applications, Treg cells will require ex vivo expansion. Expansion approaches that provide strong TCR signals tend to preferentially expand Tconv cells at the expense of Treg cells. Thus, it is essential to either initiate the expansion culture with highly purified Treg cells or create culture conditions that favor Treg cell outgrowth. Most preclinical human data analyzing human Treg cells have been generated with research-grade materials and antibody-coated magnetic beads or high-speed cell sorting. As such, these approaches have proven insufficiently robust for exportation to most institutions for Treg cell isolation under GMP conditions because of the unavailability of equivalent GMP reagents and paucity of GMP-compliant cell sorters.

The FDA mandates that the sterility, identity, purity, and potency of a cell therapy product be demonstrated before administration to patients. Sterility is the most straightforward quality to demonstrate, because assays performed on Tconv cell populations can be directly translated to Treg cells. Likewise, tracking measures, such as HLA verification and barcoding of reagents and plastics that come in contact with a particular cell product, employed to establish identity of one patient's cell product as it moves through the various processing, expansion, and validation steps prior to being reinfused require no adaptation for use in Treg cells. However, demonstrations of Treg cell purity and potency are more problematic. Foxp3 expression is a good surrogate for Treg cell purity, but given

current expansion techniques, it is unlikely that Treg cell cultures will be 100% Foxp3$^+$ after expansion. It will be important to determine "acceptable" amounts of contamination, from both quantitative and qualitative perspectives, which may be disease dependent. For reasonably well-controlled autoimmune diseases such as T1D, the acceptable proportion of non-Foxp3-expressing cells will be lower than the acceptable proportion of non-Foxp3-expressing cells in acute GVHD therapy that is a severe multiorgan system disease caused by high numbers of Tconv cells. From the qualitative perspective, it is possible, for example, that contaminating naive Tconv cells will be less problematic in autoimmune disease applications because it is unlikely that the pathogenic Tconv cells are in this subset, whereas contaminating memory cells may be less problematic in GVHD or organ transplant situations, because the alloreactive response is more likely to be contained in the naive T cell repertoire. Given these issues, it is unknown whether culture conditions capable of skewing the contaminating repertoire can be developed, especially under GMP-imposed constraints.

Monitoring of potency clearly separates Treg cells from other T cell-based therapies. For Tconv cell therapy there are in vitro surrogates, such as polyfunctional cytokine secretion by CD4$^+$ T cells and lytic potential of CD8$^+$ T cells that correlate well with in vivo efficacy (Betts et al., 2006). Unfortunately, our understanding of Treg cell function lags considerably behind our understanding of Tconv cell function. Multiple mechanisms have been implicated in Treg cell function, but there is no clear understanding of how suppression occurs in vivo (Tang and Bluestone, 2008). Although most investigators use the ability of Treg cells to inhibit T cell proliferation as a primary endpoint of in vitro suppression assays, there are many ways to measure this outcome. Suppression in some of the assays is lost when exogenous cytokines such as IL-2 are added, whereas others show suppression in the presence of IL-2. Some but not other assays are APC dependent so that if the Treg cells are acting upon APCs, suppression will be observed (Shevach, 2009, in this issue of *Immunity*). Moreover, the correlation between in vivo Treg cell activity and activity measured in vitro assays is not perfect. We recently reported that CD4$^+$ Tconv cells expanded in the presence of rapamycin were highly effective in an in vitro suppression assay, but these cells failed to function in an in vivo xeno-GVHD model (Golovina et al., 2008). Along the same lines, it is reasonable to conclude that Treg cells suppress GVHD and autoimmune disease by similar mechanisms, but this has not been formally demonstrated. Does this mean that investigators must develop disease-specific models to test the potency of expanded Treg cell products? Recent advances in tissue engineering (Azuma et al., 2007) and immune-deficient mouse models (Shultz et al., 2007; von Herrath and Nepom, 2009) have made it possible to use human Treg cells to prevent antihuman immune responses, but these models are just becoming established and their ability to predict human therapy is unproven. Considerable work is required to generate and validate fully human models of T1D, lupus, and organ transplantation.

Many of the reagents and approaches used in clinical expansion of human Treg cells can also be used with nonhuman primates (Haanstra et al., 2008; Ansari et al., 2007). Given that nonhuman primates have been instrumental in improving and validating organ transplant protocols (Lechler et al., 2005), it would seem likely that transplant studies in nonhuman primates would guide the in vivo use of human Treg cells. However, it is important to note that nonhuman primate models were used in the evaluation of the superagonistic anti-CD28 Ab TGN1412 and no toxicities were observed, calling into question the uniform utility of nonhuman primate models for Treg cell therapy (Schraven and Kalinke, 2008). Understanding when nonhuman primate studies will be informative and appropriate will be useful in driving the clinical translation of ex vivo expanded Treg cells for some indications. In any case, during the developmental stages of Treg cell therapy, there is an urgent need for in vivo-validated in vitro Treg cell functional assays so that the quality of expanded Treg cell cultures can be quickly and accurately assessed.

Current Phase I Clinical Trials Evaluating Adoptively Transferred Treg Cells

Allogeneic Treg cells have been infused into HSCT recipients in Germany and the US. These studies both utilized antibody-coated magnetic-bead separation techniques to first deplete non-T cells (B cells ± monocytes; NK cells), followed by a positive selection step to enrich for CD25$^+$ cells via a subsaturating concentration of CD25 antibody to capture the CD25^{hi} fraction (Figure 1; Godfrey et al., 2004, 2005). The final isolation products contained ~50% Treg cells as assessed by CD4, CD25, and FoxP3 flow cytometry. Further enrichment based upon FoxP3 expression is not possible because FoxP3 detection requires permeabilization. By eliminating the ex vivo expansion step, Edinger and coworkers initiated a trial in which freshly isolated allogeneic donor Treg cells are infused, avoiding the preferential expansion of contaminating Tconv cells (M. Edinger, personal communication). These fresh Treg cells were administered in a post-transplant setting to HSCT recipients who also were given a donor lymphocyte infusion to prevent or treat recurrent hematological malignancies. In concurrent phase I dose escalation trials at the University of Minnesota conducted by the coauthors and their colleagues, umbilical cord blood (UCB) was used for Treg cell isolation because cord blood is virtually devoid of memory T cells (Figure 1). In ongoing trials, nonmyeloablated or myeloablated recipients of two unrelated UCB units used for hematopoietic reconstitution, given with standard of care GVHD prophylactic pharmacological agents (cyclosporine A; mycophenolate mofetil), also received third-party, HLA partially matched Treg cells. Although CD25^{hi} Treg cells are more readily purified from umbilical cord blood than from peripheral blood (Godfrey et al., 2004, 2005), only ~5–7.5 × 10^6 Treg cells can be isolated from a frozen UCB unit. Thus, to achieve high Treg cell:Tconv cell ratios, cord blood Treg cells used in this study were expanded with anti-CD3 and anti-CD28-coated microbeads and IL-2, resulting in ~200- to 300-fold expansion in ≤3 weeks. The ability to expand peripheral blood Treg cells was advanced by the discovery that the immunosuppressant rapamycin selectively expands or preferentially preserves Treg cells over Tconv cells (Battaglia et al., 2005) because of the FoxP3-induced expression of Pim2, a serine-threonine kinase that confers rapamycin resistance (Basu et al., 2008). However, it should be noted that Treg cells are not immune to the effects of rapamycin—just less affected

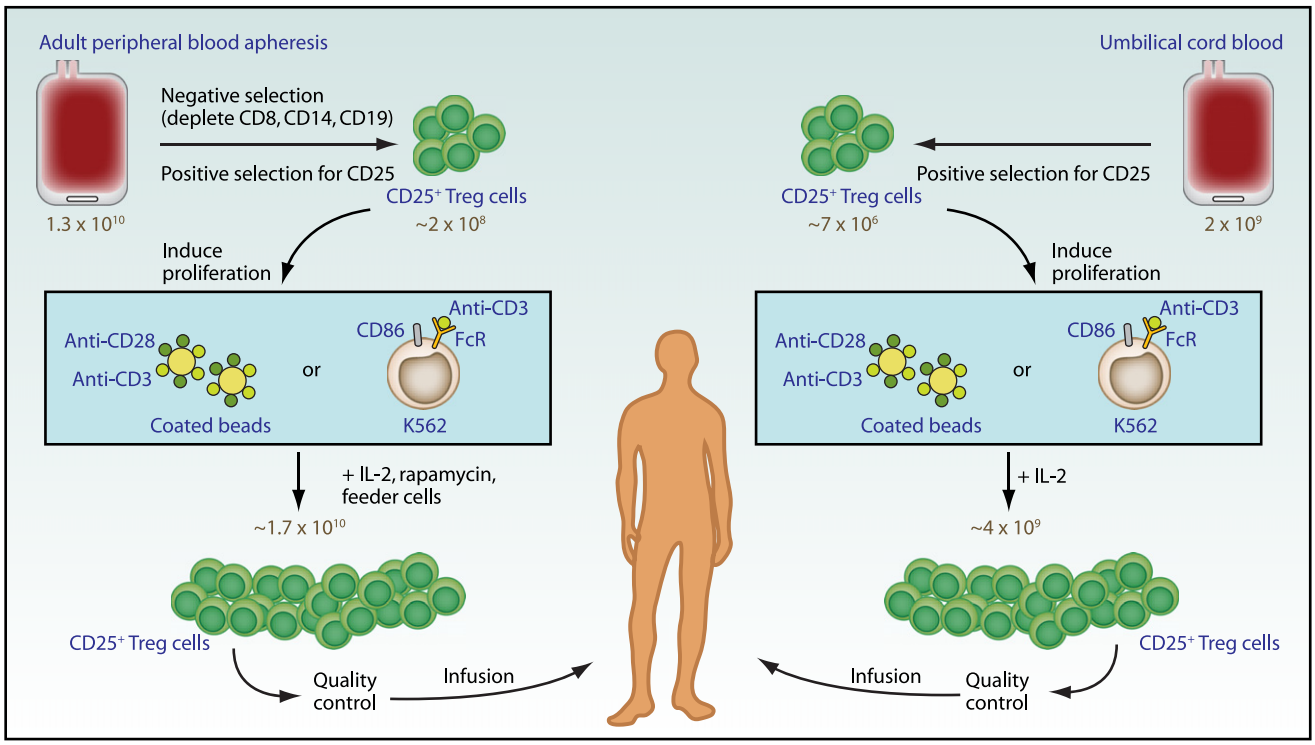

Figure 1. Clinical Applications of Human Treg Cells in Allo-HSCT

As a source of peripheral blood Treg cells, a nonmobilized apheresis unit is obtained from the HSCT donor ~18–20 days prior to transplant (left). Non-Treg cell populations are depleted of CD8, CD14, and CD19 with either magnetic beads or flow cytometry techniques. Alternatively, a third-party UCB unit is used that does not require negative selection (right). Treg cells are enriched by CD25-positive selection. These populations can be expanded with CD3 and CD28 mAb-coated microbeads or a cell-based aAPC consisting of K562 cells transduced to express CD86 and an FcR (CD32 or CD64) upon which CD3 mAb is loaded. Exogenous IL-2, rapamycin, and an irradiated $CD4^+CD25^-$ feeder layer were added to the culture for peripheral blood Treg cell expansion, whereas UCB Treg cell expansion required only supplemental IL-2. After quality control studies are completed, Treg cells are infused in the peri-HSCT period either in the context of GVHD prophylactic drugs or T cell mAb or in vitro T cell depletion. The approximate cell yields at each step are listed.

than Tconv cells. Thus, multiple ex vivo stimulations may be required to achieve therapeutic doses of Treg cells (Golovina et al., 2008).

As noted above, UCB Treg cells are an attractive therapeutic modality. They are largely naive, have long telomeres, and are easily separated from Tconv cells because of the reduced complexity of UCB T cell subsets. One drawback is that to date UCB has been employed as an allogeneic cell source, necessitating HLA matching and opening the possibility of host versus graft or graft versus host disease. However, within the last decade or so, banking of infant UCB has increased markedly, so it is possible to envision autologous UCB therapy and in particular, therapy with ex vivo expanded autologous UCB-derived Treg cells. Investigators at the University of Florida (Haller, Atkinson, and Schatz) have infused 23 T1D individuals with autologous, unfractionated UCB (M.A. Atkinson, personal communication). Because UCB contains mesenchymal stem cells, Treg cells, and perhaps other undefined cells with suppressor activity, it will be difficult to ascribe any therapeutic benefit to a particular cell subset. Nonetheless, this trial establishes the safety, and rather impressively, the feasibility of using autologous UCB to treat autoimmune disease, opening many exciting possibilities for future trials. Thus, it is possible to envision future approaches with similar advantages of UCB with autologous Treg cells derived from induced pluripotent stem cells.

Potential Dangers of Human Treg Cell Therapy

Like all therapies, clinical use of ex vivo expanded Treg cells is associated with potential risks. Perhaps the most troublesome is the possibility of expanded Treg cells reverting to Tconv cells, especially if antigen-specific Treg cells are infused. The notion of plasticity among the various T cell subsets has gained much attention (Zhou et al., 2009, in this issue of *Immunity*), driven in part by studies with $Foxp3^{EGFP}$ knockin mice (Fontenot et al., 2005). One surprising and intriguing finding from this line of research is that TGF-β is required for both Treg and Th17 cell differentiation, with the ultimate fate decision resting on interactions between the transcription factors SMAD4, RORγ, RORα, STAT3, and the cytokines IL-1 and IL-6 (Yang et al., 2008; Radhakrishnan et al., 2008). The majority of adoptively transferred Treg cells maintain their suppressive activity, but a minority of cells lose Foxp3 expression and can differentiate into Tconv cells (Komatsu et al., 2009). Understanding why cells lose their "Treg-ness" and preventing this dedifferentation in vivo will improve both the safety and efficacy of Treg cell therapy. Because Foxp3 is the master regulator of Treg cell function (Josefowicz and Rudensky, 2009, in this issue of *Immunity*), alterations in Foxp3 expression or activity are likely involved in converting Treg cells to Tconv cells. Foxp3 expression is modulated by DNA methylation via CpG islands in its promoter (Kim and Leonard, 2007) and by chromatin remodeling (Tao et al., 2007).

Therefore, administration of selective demethylation agents and/or histone protein deacetylases (HDACs) may enhance Treg cell function and fidelity in vivo. It will be of interest to determine whether human Treg cells are as plastic as their murine counterparts when given in vivo for therapeutic intent.

There are also concerns that excessive Treg cell activity may blunt the response to infectious agents or lead to higher rates of tumor occurrence or relapse. These are justified concerns. However, one study showed that augmenting Treg cell activity via TGF-β administration protected NOD mice from T1D but did not prevent coxsackievirus clearance (Richer et al., 2008). Clearly, more studies are required to examine whether therapeutic levels of adoptively transferred Treg cells restrict protective immune responses. Although the use of suicide vectors may be an attractive way to eliminate introduced Treg cells if excessive immunosuppression is observed, this would likely require some form of gene therapy, which requires its own careful assessment of risks and rewards.

Future Directions for Treg Cells in the Clinic

Treg cells are not a homogeneous population. Like their Tconv cell counterparts, they can be divided into subsets based on differential cell surface marker expression (Gajewski, 2007). The functional implications of these differences are now becoming apparent. For example, $ICOS^+$ $Foxp3^+$ Treg cells produce more IL-10 than do their $ICOS^-$ $Foxp3^+$ counterparts and it has been suggested that they play a more important role in modulating dendritic cell function (Ito et al., 2008). Most adoptive T cell therapy studies to date have used $CD4^+CD25^{2+}$ or $CD4^+CD25^+CD127^-$ cells, but now that it is clear that human Treg cell subsets exist, it is essential to determine whether one particular subset(s) is more attractive for therapeutic use. It is possible that Treg cell subset appropriateness will vary with the application. Perhaps distinct Treg cell subsets will be best suited for GVHD applications, whereas other subsets may be better suited to control autoimmune diseases, perhaps dependent upon trafficking patterns or relative survival in a particular niche. Understanding how chemokines and integrins (Wei et al., 2006) control the migration and perhaps survival of distinct Treg cell subsets will enable informed decisions concerning which, if any, Treg cell subset to employ for a particular clinical application.

After antigen encounter, a subset of naive Tconv cells becomes memory T cells. Do memory Treg cells exist, and if so, would these antigen-experienced cells respond more rapidly and vigorously to antigen? A study examining MS patients revealed that the number of $CD31^+$ cells coexpressing CD4, CD25, CD45RA, and Foxp3 declines with age in healthy controls and this decline is more severe in MS patients (Haas et al., 2007). Although these data suggest that maintaining a pool of naive Treg cells is important, it is important to note that the existence of Treg memory cells has not been demonstrated. Because Treg cells appear to retain expression of CCR7, CD62L, CD28, and CD27, but do not express CD127 (Liu et al., 2006; Hoffmann et al., 2006; Godfrey et al., 2005), it is unclear how Treg memory cell subsets can best be defined phenotypically. Both $CD45RA^+CD25^{2+}$ and $CD45RO^+CD25^{2+}$ Treg cells exist, but it is not clear whether the CD45RO cells represent contaminating Tconv or Treg cells. In any case, $CD45RO^+CD25^{2+}$ cells exhibited significantly less suppressive activity after expansion than did $CD45RA^+CD25^{2+}$ cells (Hoffmann et al., 2006), suggesting that these cells do not have the functional properties of memory Treg cells.

As evident from our title, it is unclear what constitutes a therapeutic dose of Treg cells. The answer depends on many factors, including the specific disease targeted and whether polyclonal or antigen-specific Treg cells are employed. Defining Treg cell dosing strategies requires large-scale Treg cell expansion capacity. It has been reported that with a murine cell-based artificial APC (aAPC) system, human Treg cells could be expanded 40,000-fold in 3–4 weeks (Hoffmann et al., 2004). Unfortunately, this study preceded the development of robust Foxp3 staining protocols and in vivo models of Treg cell function, so the purity and potency of these Treg cells is difficult to ascertain. Subsequent studies have reported more modest expansion of Treg cells with anti-CD3- and anti-CD28-coated beads (of which GMP versions are available) in the presence of high amounts of IL-2 (Earle et al., 2005; Godfrey et al., 2004). We have found that anti-CD3 Ab-loaded K562-based aAPCs expand Treg cells more efficiently, and the expanded cells exhibit greater purity and potency than do cells expanded with anti-CD3 and anti-CD28 beads (Golovina et al., 2008; Hippen et al., 2008). Moreover, when using these aAPCs, a 1000-fold expansion in ~3 weeks is achievable if two stimulations are employed, making it feasible to propose dosing at numbers comparable to those used in $CD4^+$ Tconv cell adoptive transfer applications. Another advantage of K562-based aAPCs is that additional cell surface (costimulatory molecules) and secreted (cytokines and chemokines) molecules can be easily added to further refine human Treg cell expansion (Suhoski et al., 2007). To date, our data with human isolated from peripheral blood indicate that addition of costimulatory ligands other than CD86 favors the expansion of contaminating Tconv cells at the expense of Treg cells (Golovina et al., 2008). In contrast, expansion of UCB Treg cells can be enhanced by the presence of additional costimulatory ligands (Hippen et al., 2008). This discrepancy is most likely a consequence of the substantially higher purity of the starting Treg cell populations from UCB. Lastly, because K562 cells are of human origin, and irradiated K562 variants expressing GM-CSF have been safely injected into humans (Nemunaitis et al., 2006), this raises the possibility of in vivo Treg cell expansion with irradiated K562-based aAPCs.

The ability to overcome some of the limitations of Treg cell therapy by genetically reprogramming Treg cells is potentially attractive, especially in light of the availability of clinical grade lentiviral vectors (Levine et al., 2006). One area of active research is to use vectors to redirect polyclonal Treg cells to specific targets. In murine models of T1D, antigen-specific Treg cells are far more potent than polyclonal Treg cells on a per-cell basis (Tang et al., 2004). However, current technology does not easily permit the identification of islet-specific Treg cells in humans. Moreover, multiple rounds of expansion would likely be required to obtain therapeutic numbers of Treg cells, which might compromise their engraftment, persistence, or function. In murine models, introduction of a chimeric immune receptor (CIR) into Treg cells prevented experimental autoimmune encephalomyelitis (Mekala and Geiger, 2005) and experimental-induced colitis (Eran et al., 2009). Likewise, recent advances in engineering and expressing TCRs that redirect T cell specificity (June, 2007) could also be

applied to improve TCR-transduced Treg cell therapy (Hori et al., 2002). We recently demonstrated that high-affinity TCRs conferred greater effector function to Tconv cells (Varela-Rohena et al., 2008). It will be interesting to determine whether this applies to Treg cells as well. Additional gene modifications can be envisioned that may enhance the therapeutic potential of Treg cells. The FoxP3 deficiency in IPEX patients could be corrected in T cells, or even better in hematopoietic stem cells, by either zinc-finger nuclease-mediated repair for a point mutation or small insertions/deletions (Urnov et al., 2005) or lentiviral vector-mediated introduction of the wild-type Foxp3 gene under the control of a Treg cell-specific promoter. Likewise, to prevent the Treg-Tconv cell conversion, Treg cells could be engineered to constitutively overexpress Foxp3 so that these infused Treg cells are less likely to convert to Tconv cells. Lastly, as our knowledge of Treg cell immune-suppressive mechanisms increases, additional opportunities to enhance Treg cell function via gene therapy will present themselves.

Because natural Treg cells are present at a low frequency in peripheral blood, CD25-based purification techniques remove the vast majority of $CD4^+$ T cells. Because $CD4^+CD25^-$ T cells can be induced to express high amounts of FoxP3, an alternative approach to natural Treg cell purification is to generate inducible Treg cells (iTreg cells) by subjecting $CD4^+CD25^-$ T cells to conditions that result in the gain of suppressor cell function (Curotto de Lafaille and Lafaille, 2009, in this issue of *Immunity*). Although some species-specific differences are likely to exist, murine iTreg cells can be generated after ex vivo exposure of naive $CD4^+CD25^-$ or $CD4^+CD45RO^-$ T cells to TGF-β (Chen et al., 2003), especially in the presence of IL-2 or IL-10, vitamin D3 (Barrat et al., 2002), all trans-retinoic acid (Benson et al., 2007), indoleamine 2,3 dioxygenase (Chen et al., 2008), or FoxP3-expressing retroviruses (Hori et al., 2003). Other strategies for in vitro iTreg cell generation may include exposing $CD4^+CD25^-$ T cells to subimmunogenic antigen-loaded dendritic cells or to histone deacetylase inhibitors that specifically regulate FoxP3 expression. Although potentially promising, there is a paucity of data regarding the yield, stability, plasticity, and relative potency of iTreg versus natural Treg cells as assessed by both in vitro and in vivo measures. Thus, it would be premature to gauge whether iTreg cell generation may circumvent the technical limitations of producing high numbers of natural Treg cells via GMP reagents.

Concluding Statements

It is becoming increasingly clear that a major component of the next wave of therapeutic agents that will attempt to tackle our unmet medical needs will be cell and gene therapy. When this wave will hit the clinics and become the standard of care for many disease states is far away, but we are convinced that it will happen because cells and in particular Treg cells offer the ability to be highly specific and strikingly effective. Also, Treg cell therapy has the promise of avoiding many of the toxicities observed with current drug regimens. An argument can be made that this potential was realized perhaps too early, before we really had a reasonable understanding of the checks and balances the immune system employs to maintain tolerance and promote immunity. Many of the initial attempts at Treg cell therapy used inferior cell expansion systems that did not produce cells with a high engraftment potential, and many of the gene therapy products proved to be highly immunogenic and were rapidly cleared from the body. We are now poised to enter the next generation of cell and gene therapy, armed with volumes of basic research and preclinical testing. Will this information be sufficient to unlock the power of Treg cells and provide better options for those suffering from autoimmune and other immune-mediated disease states? Only time and hard work will answer this question but nonetheless we envision that one day, as our title implies, Treg cell therapy will be as commonplace as taking two aspirins.

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REFERENCES

Anderson, M.S., and Bluestone, J.A. (2005). THE NOD MOUSE: A model of immune dysregulation. Annu. Rev. Immunol. *23*, 447–485.

Anderson, B.E., McNiff, J.M., Matte, C., Athanasiadis, I., Shlomchik, W.D., and Shlomchik, M.J. (2004). Recipient CD4+ T cells that survive irradiation regulate chronic graft-versus-host disease. Blood *104*, 1565–1573.

Ansari, A.A., Pereira, L.E., Mayne, A.E., Onlamoon, N., Pattanapanyasat, K., Mori, K., and Villinger, F. (2007). The role of disease stage, plasma viral load and regulatory T cells (Tregs) on autoantibody production in SIV-infected non-human primates. J. Autoimmun. *28*, 152–159.

Azuma, H., Paulk, N., Ranade, A., Dorrell, C., Al-Dhalimy, M., Ellis, E., Strom, S., Kay, M.A., Finegold, M., and Grompe, M. (2007). Robust expansion of human hepatocytes in Fah−/−/Rag2−/−/Il2rg−/− mice. Nat. Biotechnol. *25*, 903–910.

Baecher-Allan, C., and Hafler, D.A. (2006). Human regulatory T cells and their role in autoimmune disease. Immunol. Rev. *212*, 203–216.

Barrat, F.J., Cua, D.J., Boonstra, A., Richards, D.F., Crain, C., Savelkoul, H.F., de Waal-Malefyt, R., Coffman, R.L., Hawrylowicz, C.M., and O'Garra, A. (2002). In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J. Exp. Med. *195*, 603–616.

Basu, S., Golovina, T., Mikheeva, T., June, C.H., and Riley, J.L. (2008). Cutting edge: Foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin. J. Immunol. *180*, 5794–5798.

Battaglia, M., Stabilini, A., and Roncarolo, M.G. (2005). Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood *105*, 4743–4748.

Battaglia, M., Stabilini, A., Migliavacca, B., Horejs-Hoeck, J., Kaupper, T., and Roncarolo, M.G. (2006). Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. J. Immunol. *177*, 8338–8347.

Benson, M.J., Pino-Lagos, K., Rosemblatt, M., and Noelle, R.J. (2007). All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J. Exp. Med. *204*, 1765–1774.

Betts, M.R., Nason, M.C., West, S.M., De Rosa, S.C., Migueles, S.A., Abraham, J., Lederman, M.M., Benito, J.M., Goepfert, P.A., Connors, M., et al. (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood *107*, 4781–4789.

Beyersdorf, N., Gaupp, S., Balbach, K., Schmidt, J., Toyka, K.V., Lin, C.H., Hanke, T., Hunig, T., Kerkau, T., and Gold, R. (2005). Selective targeting of

regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. J. Exp. Med. *202*, 445–455.

Brusko, T., Wasserfall, C., McGrail, K., Schatz, R., Viener, H.L., Schatz, D., Haller, M., Rockell, J., Gottlieb, P., Clare-Salzler, M., and Atkinson, M. (2007). No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes *56*, 604–612.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+ CD25-naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J. Exp. Med. *198*, 1875–1886.

Chen, Z., Herman, A.E., Matos, M., Mathis, D., and Benoist, C. (2005). Where CD4+CD25+ T reg cells impinge on autoimmune diabetes. J. Exp. Med. *202*, 1387–1397.

Chen, W., Liang, X., Peterson, A.J., Munn, D.H., and Blazar, B.R. (2008). The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J. Immunol. *181*, 5396–5404.

Christen, U., and Herrath, M.G. (2004). Initiation of autoimmunity. Curr. Opin. Immunol. *16*, 759–767.

Cohen, J.L., Trenado, A., Vasey, D., Klatzmann, D., and Salomon, B.L. (2002). CD4(+)CD25(+) immunoregulatory T cells: New therapeutics for graft-versus-host disease. J. Exp. Med. *196*, 401–406.

Crispin, J.C., Martinez, A., and Alcocer-Varela, J. (2003). Quantification of regulatory T cells in patients with systemic lupus erythematosus. J. Autoimmun. *21*, 273–276.

Curotto de Lafaille, M.A., and Lafaille, J.J. (2009). Natural and adaptive Foxp3+ regulatory T cells: More of the same, or a division of labor? Immunity *30*, this issue, 626–635.

Davies, J.D., O'Connor, E., Hall, D., Krahl, T., Trotter, J., and Sarvetnick, N. (1999). CD4+ CD45RB low-density cells from untreated mice prevent acute allograft rejection. J. Immunol. *163*, 5353–5357.

Earle, K.E., Tang, Q., Zhou, X., Liu, W., Zhu, S., Bonyhadi, M.L., and Bluestone, J.A. (2005). In vitro expanded human CD4+CD25+ regulatory T cells suppress effector T cell proliferation. Clin. Immunol. *115*, 3–9.

Egen, J.G., Kuhns, M.S., and Allison, J.P. (2002). CTLA-4: New insights into its biological function and use in tumor immunotherapy. Nat. Immunol. *3*, 611–618.

Eran, E., Nitzan, A., Tova, W., and Eshhar, Z. (2009). Amelioration of colitis by genetically engineered murine regulatory T cells redirected by antigen-specific chimeric receptor. Gastroenterology, in press.

Flores-Borja, F., Jury, E.C., Mauri, C., and Ehrenstein, M.R. (2008). Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc. Natl. Acad. Sci. USA *105*, 19396–19401.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005). Regulatory T cell lineage specification by the Forkhead transcription factor Foxp3. Immunity *22*, 329–341.

Friedline, R.H., Brown, D.S., Nguyen, H., Kornfeld, H., Lee, J., Zhang, Y., Appleby, M., Der, S.D., Kang, J., and Chambers, C.A. (2009). CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. J. Exp. Med. *206*, 421–434.

Gajewski, T.F. (2007). The expanding universe of regulatory T cell subsets in cancer. Immunity *27*, 185–187.

Gershon, R.K. (1975). A disquisition on suppressor T cells. Transplant. Rev. *26*, 170–185.

Godfrey, W.R., Ge, Y.G., Spoden, D.J., Levine, B.L., June, C.H., Blazar, B.R., and Porter, S.B. (2004). In vitro-expanded human CD4(+)CD25(+) T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. Blood *104*, 453–461.

Godfrey, W.R., Spoden, D.J., Ge, Y.G., Baker, S.R., Liu, B., Levine, B.L., June, C.H., Blazar, B.R., and Porter, S.B. (2005). Cord blood CD4(+)CD25(+)-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. Blood *105*, 750–758.

Golovina, T.N., Mikheeva, T., Suhoski, M.M., Aqui, N.A., Tai, V.C., Shan, X., Liu, R., Balcarcel, R.R., Fisher, N., Levine, B.L., et al. (2008). CD28 costimulation is essential for human T regulatory expansion and function. J. Immunol. *181*, 2855–2868.

Haanstra, K.G., van der Maas, M.J., 't Hart, B.A., and Jonker, M. (2008). Characterization of naturally occurring CD4+CD25+ regulatory T cells in rhesus monkeys. Transplantation *85*, 1185–1192.

Haas, J., Hug, A., Viehover, A., Fritzsching, B., Falk, C.S., Filser, A., Vetter, T., Milkova, L., Korporal, M., Fritz, B., et al. (2005). Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur. J. Immunol. *35*, 3343–3352.

Haas, J., Fritzsching, B., Trubswetter, P., Korporal, M., Milkova, L., Fritz, B., Vobis, D., Krammer, P.H., Suri-Payer, E., and Wildemann, B. (2007). Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. J. Immunol. *179*, 1322–1330.

Hall, B.M., Pearce, N.W., Gurley, K.E., and Dorsch, S.E. (1990). Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. J. Exp. Med. *171*, 141–157.

Hanash, A.M., and Levy, R.B. (2005). Donor CD4+CD25+ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. Blood *105*, 1828–1836.

Hippen, K.L., Harker-Murray, P., Porter, S.B., Merkel, S.C., Londer, A., Taylor, D.K., Bina, M., Panoskaltsis-Mortari, A., Rubinstein, P., Van, R.N., et al. (2008). Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4–1BB expressed on artificial antigen-presenting cells. Blood *112*, 2847–2857.

Hoffmann, P., Ermann, J., Edinger, M., Fathman, C.G., and Strober, S. (2002). Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J. Exp. Med. *196*, 389–399.

Hoffmann, P., Eder, R., Kunz-Schughart, L.A., Andreesen, R., and Edinger, M. (2004). Large-scale in vitro expansion of polyclonal human CD4+CD25high regulatory T cells. Blood *104*, 895–903.

Hoffmann, P., Eder, R., Boeld, T.J., Doser, K., Piseshka, B., Andreesen, R., and Edinger, M. (2006). Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. Blood *108*, 4260–4267.

Hori, S., Haury, M., Coutinho, A., and Demengeot, J. (2002). Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. Proc. Natl. Acad. Sci. USA *99*, 8213–8218.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057–1061.

Ito, T., Hanabuchi, S., Wang, Y.H., Park, W.R., Arima, K., Bover, L., Qin, F.X.-F., Gilliet, M., and Liu, Y.J. (2008). Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity *28*, 870–880.

Jarvinen, L.Z., Blazar, B.R., Adeyi, O.A., Strom, T.B., and Noelle, R.J. (2003). CD154 on the surface of CD4+CD25+ regulatory T cells contributes to skin transplant tolerance. Transplantation *76*, 1375–1379.

Joffre, O., Gorsse, N., Romagnoli, P., Hudrisier, D., and van Meerwijk, J.P. (2004). Induction of antigen-specific tolerance to bone marrow allografts with CD4+CD25+ T lymphocytes. Blood *103*, 4216–4221.

Josefowicz, S.Z., and Rudensky, A. (2009). Control of the regulatory T cell lineage commitment and maintenance. Immunity *30*, this issue, 616–625.

June, C.H. (2007). Principles of adoptive T cell cancer therapy. J. Clin. Invest. *117*, 1204–1212.

June, C.H., and Blazar, B.R. (2006). Clinical application of expanded CD4+25+ cells. Semin. Immunol. *18*, 78–88.

Kim, H.P., and Leonard, W.J. (2007). CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: A role for DNA methylation. J. Exp. Med. *204*, 1543–1551.

Komatsu, N., Mariotti-Ferrandiz, M.E., Wang, Y., Malissen, B., Waldmann, H., and Hori, S. (2009). Heterogeneity of natural Foxp3+ T cells: A committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. Proc. Natl. Acad. Sci. USA *106*, 1903–1908.

Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T.R., Backstrom, B.T., Sobel, R.A., Wucherpfennig, K.W., Strom, T.B., et al. (2007). Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat. Med. *13*, 423–431.

Koulmanda, M., Bhasin, M., Hoffman, L., Fan, Z., Qipo, A., Shi, H., Bonner-Weir, S., Putheti, P., Degauque, N., Libermann, T.A., et al. (2008). Curative and beta cell regenerative effects of alpha1-antitrypsin treatment in autoimmune diabetic NOD mice. Proc. Natl. Acad. Sci. USA *105*, 16242–16247.

Kukreja, A., Cost, G., Marker, J., Zhang, C., Sun, Z., Lin-Su, K., Ten, S., Sanz, M., Exley, M., Wilson, B., et al. (2002). Multiple immuno-regulatory defects in type-1 diabetes. J. Clin. Invest. *109*, 131–140.

Lechler, R.I., Sykes, M., Thomson, A.W., and Turka, L.A. (2005). Organ transplantation—how much of the promise has been realized? Nat. Med. *11*, 605–613.

Levine, B.L., Humeau, L.M., Boyer, J., MacGregor, R.R., Rebello, T., Lu, X., Binder, G.K., Slepushkin, V., Lemiale, F., Mascola, J.R., et al. (2006). Gene transfer in humans using a conditionally replicating lentiviral vector. Proc. Natl. Acad. Sci. USA *103*, 17372–17377.

Lewis, E.C., Mizrahi, M., Toledano, M., Defelice, N., Wright, J.L., Churg, A., Shapiro, L., and Dinarello, C.A. (2008). alpha1-Antitrypsin monotherapy induces immune tolerance during islet allograft transplantation in mice. Proc. Natl. Acad. Sci. USA *105*, 16236–16241.

Li, X., Li, X., Qian, L., Wang, G., Zhang, H., Wang, X., Chen, K., Zhai, Z., Li, Q., Wang, Y., and Harris, D.C. (2007). T regulatory cells are markedly diminished in diseased salivary glands of patients with primary Sjogren's syndrome. J. Rheumatol. *34*, 2438–2445.

Lin, C.H., and Hunig, T. (2003). Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. Eur. J. Immunol. *33*, 626–638.

Lindley, S., Dayan, C.M., Bishop, A., Roep, B.O., Peakman, M., and Tree, T.I. (2005). Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes *54*, 92–99.

Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas de St, G.B., et al. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J. Exp. Med. *203*, 1701–1711.

Mekala, D.J., and Geiger, T.L. (2005). Immunotherapy of autoimmune encephalomyelitis with redirected CD4+CD25+ T lymphocytes. Blood *105*, 2090–2092.

Miyara, M., Amoura, Z., Parizot, C., Badoual, C., Dorgham, K., Trad, S., Nochy, D., Debre, P., Piette, J.C., and Gorochov, G. (2005). Global natural regulatory T cell depletion in active systemic lupus erythematosus. J. Immunol. *175*, 8392–8400.

Nemunaitis, J., Jahan, T., Ross, H., Sterman, D., Richards, D., Fox, B., Jablons, D., Aimi, J., Lin, A., and Hege, K. (2006). Phase 1//2 trial of autologous tumor mixed with an allogeneic GVAX[reg] vaccine in advanced-stage non-small-cell lung cancer. Cancer Gene Ther. *13*, 555–562.

Olivares-Villagomez, D., Wang, Y., and Lafaille, J.J. (1998). Regulatory CD4+ T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. J. Exp. Med. *188*, 1883–1894.

Putnam, A.L., Brusko, T.M., Lee, M.R., Liu, W., Szot, G.L., Ghosh, T., Atkinson, M.A., and Bluestone, J.A. (2008). Expansion of human regulatory T cells from patients with type 1 diabetes. Diabetes *58*, 652–662.

Radhakrishnan, S., Cabrera, R., Schenk, E.L., Nava-Parada, P., Bell, M.P., Van Keulen, V.P., Marler, R.J., Felts, S.J., and Pease, L.R. (2008). Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. J. Immunol. *181*, 3137–3147.

Richer, M.J., Straka, N., Fang, D., Shanina, I., and Horwitz, M.S. (2008). Regulatory T-cells protect from type 1 diabetes after induction by coxsackievirus infection in the context of transforming growth factor-{beta}. Diabetes *57*, 1302–1311.

Riley, J.L., and June, C.H. (2005). The CD28 family: A T-cell rheostat for therapeutic control of T-cell activation. Blood *105*, 13–21.

Rosenberg, A.S., and Singer, A. (1992). Cellular basis of skin allograft rejection: An in vivo model of immune-mediated tissue destruction. Annu. Rev. Immunol. *10*, 333–358.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. *155*, 1151–1164.

Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. (2006). Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol. Rev. *212*, 8–27.

Sanchez-Fueyo, A., Weber, M., Domenig, C., Strom, T.B., and Zheng, X.X. (2002). Tracking the immunoregulatory mechanisms active during allograft tolerance. J. Immunol. *168*, 2274–2281.

Scalapino, K.J., and Daikh, D.I. (2008). CTLA-4: A key regulatory point in the control of autoimmune disease. Immunol. Rev. *223*, 143–155.

Schneider, A., Rieck, M., Sanda, S., Pihoker, C., Greenbaum, C., and Buckner, J.H. (2008). The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. J. Immunol. *181*, 7350–7355.

Schraven, B., and Kalinke, U. (2008). CD28 superagonists: What makes the difference in humans? Immunity *28*, 591–595.

Shevach, E.M. (2009). Mechanisms of Foxp3+ T regulatory cell-mediated suppression. Immunity *30*, this issue, 636–645.

Shultz, L.D., Ishikawa, F., and Greiner, D.L. (2007). Humanized mice in translational biomedical research. Nat. Rev. Immunol. *7*, 118–130.

Suhoski, M.M., Golovina, T.N., Aqui, N.A., Tai, V.C., Varela-Rohena, A., Milone, M.C., Carroll, R.G., Riley, J.L., and June, C.H. (2007). Engineering artificial antigen-presenting cells to express a diverse array of co-stimulatory molecules. Mol. Ther. *15*, 981–988.

Suntharalingam, G., Perry, M.R., Ward, S., Brett, S.J., Castello-Cortes, A., Brunner, M.D., and Panoskaltsis, N. (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N. Engl. J. Med. *355*, 1018–1028.

Szanya, V., Ermann, J., Taylor, C., Holness, C., and Fathman, C.G. (2002). The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. J. Immunol. *169*, 2461–2465.

Tang, Q., and Bluestone, J.A. (2008). The Foxp3+ regulatory T cell: A jack of all trades, master of regulation. Nat. Immunol. *9*, 239–244.

Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., and Bluestone, J.A. (2004). In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J. Exp. Med. *199*, 1455–1465.

Tao, R., de Zoeten, E.F., Ozkaynak, E., Chen, C., Wang, L., Porrett, P.M., Li, B., Turka, L.A., Olson, E.N., Greene, M.I., et al. (2007). Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat. Med. *13*, 1299–1307.

Tarbell, K.V., Petit, L., Zuo, X., Toy, P., Luo, X., Mqadmi, A., Yang, H., Suthanthiran, M., Mojsov, S., and Steinman, R.M. (2007). Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. J. Exp. Med. *204*, 191–201.

Taylor, P.A., Noelle, R.J., and Blazar, B.R. (2001). CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. J. Exp. Med. *193*, 1311–1318.

Taylor, P.A., Lees, C.J., and Blazar, B.R. (2002). The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood *99*, 3493–3499.

Taylor, P.A., Panoskaltsis-Mortari, A., Swedin, J.M., Lucas, P.J., Gress, R.E., Levine, B.L., June, C.H., Serody, J.S., and Blazar, B.R. (2004). L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood *104*, 3804–3812.

Trenado, A., Charlotte, F., Fisson, S., Yagello, M., Klatzmann, D., Salomon, B.L., and Cohen, J.L. (2003). Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. J. Clin. Invest. *112*, 1688–1696.

Urnov, F.D., Miller, J.C., Lee, Y.L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D., and Holmes, M.C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature *435*, 646–651.

Valencia, X., Yarboro, C., Illei, G., and Lipsky, P.E. (2007). Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. J. Immunol. *178*, 2579–2588.

Varela-Rohena, A., Molloy, P.E., Dunn, S.M., Li, Y., Suhoski, M.M., Carroll, R.G., Milicic, A., Mahon, T., Sutton, D.H., Laugel, B., et al. (2008). Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. Nat. Med. *14*, 1390–1395.

Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J. Exp. Med. *199*, 971–979.

von Herrath, M., and Nepom, G.T. (2009). Animal models of human type 1 diabetes. Nat. Immunol. *10*, 129–132.

Wei, S., Kryczek, I., and Zou, W. (2006). Regulatory T-cell compartmentalization and trafficking. Blood *108*, 426–431.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science *322*, 271–275.

Wood, K.J., and Sakaguchi, S. (2003). Regulatory T cells in transplantation tolerance. Nat. Rev. Immunol. *3*, 199–210.

Yang, X.O., Nurieva, R., Martinez, G.J., Kang, H.S., Chung, Y., Pappu, B.P., Shah, B., Chang, S.H., Schluns, K.S., Watowich, S.S., et al. (2008). Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity *29*, 44–56.

Zhao, D., Zhang, C., Yi, T., Lin, C.L., Todorov, I., Kandeel, F., Forman, S., and Zeng, D. (2008). In vivo-activated CD103+CD4+ regulatory T cells ameliorate ongoing chronic graft-versus-host disease. Blood *112*, 2129–2138.

Zhou, L., Chong, M.M.W., and Littman, D.R. (2009). Plasticity of CD4+ T cell lineage differentiation. Immunity *30*, this issue, 646–655.