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Human T Regulatory Cells as Therapeutic Agents: Take a Billion or So of These and Call Me in the Morning

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Summary

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 cells (Tregs) 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 Tregs can prevent both local and systemic organ and tissue destruction. Although promising, human Treg 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 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 non-redundant regulatory mechanisms exist to maintain this delicate balance. The focus of this review stems from the seminal work of Sakaguchi et al. These studies reported that 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 cells, [Tregs]). The importance of Tregs in immune regulation has been well-established in mice (Asano et al., 1996; Takahashi et al., 1998; Suri-Payer et al., 1998; Thornton and Shevach, 1998) and humans (Jonuleit et al., 2001; Baecher-Allan et al., 2001; Dieckmann et al., 2001). While the therapeutic potential of Tregs was envisioned decades ago (Gershon, 1975), clinical implementation of their potent immune regulatory activity has proven challenging. Like conventional T cells (Tconvs), Tregs need TCR triggering and costimulation to become fully active. Although some costimulatory pathways may differentially regulate Tregs 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 activity may also augment Tconv activity. This point was most dramatically illustrated in a Phase I clinical trial testing TGN1412, a superagonistic anti-CD28 antibody (Ab) (Suntharalingam et al., 2006). Preclinical data in animal models demonstrated that this Ab preferentially activated Tregs, and it was thus postulated that TGN1412 could restore tolerance and enable transplants (Beyersdorf et al., 2005; Lin and Hunig, 2003; Beyersdorf et al.,

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2006). Unfortunately, when seemingly low Ab doses were delivered to 6 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 non-human 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 Tregs (June and Blazar, 2006). In this approach, Tregs 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 Tregs 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 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 Tregs 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 activation consequent to diminished Treg 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 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 Tregs to treat autoimmune disease associated with the loss of CTLA-4 activity is stronger if Treg function is reasonably preserved and autoimmunity is due to higher numbers of autoreactive T cells. Here, the addition of functional Tregs may reset the proper threshold between tolerance and immunity. If CTLA-4 activity is required for Treg effector functions, then the rationale of infusing additional non-fully functional Tregs is not so clear.

The relative contributions of reduced Treg numbers and reduced Treg activity to autoimmunity is an important unresolved issue. While reduced numbers of Tregs 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 Tregs (Putnam et al., 2008; Brusko et al., 2007; Lindley et al., 2005). Although freshly isolated T1D patient Tregs have been reported to be less effective suppressors than Tregs from healthy control donors, it was not clear from these studies whether the Tregs were less effective or the responder Tconv cells were more resistant to suppression (Glisic-Milosavljevic et al., 2007; Lindley et al., 2005). Importantly, Tregs from a T1D patient, grown ex vivo using a suboptimal T cell expansion system incorporating rapamycin, a potent pharmacological inhibitor of Tconv responses, suppressed allogeneic Tconvs as potently as similarly cultured Tregs from healthy controls. This suggested that functional deficits in Tregs from T1D patients were corrected

during the expansion process (Battaglia et al., 2006). This suggestion is supported by recent data indicating that Tregs 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 molecular defects underlying given autoimmune diseases may guide the decision to pursue immunotherapy with adoptively transferred Tregs.

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 conventional $CD4^+$ and/or $CD8^+$ T cells (Tconvs) 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). Following 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.

Following 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 anti-host alloresponses that culminate in the multi-organ system disorder known as graft-versus-host disease (GVHD) typically is highest in the first 1–3 months post-HSCT. 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 Tregs can be isolated from healthy HSCT donors, human Treg trials in HSCT recipients have preceded other indications.

Preclinical data in support for Treg infusional therapy in autoimmunity

T1D results from the failure to control islet-specific Tconvs (Tisch and McDevitt, 1996; Roep, 2003). The role of Tregs in the establishment and progression of T1D has been intensely studied. Non-obese diabetic (NOD) mice spontaneously acquire T1D and they have been the workhorse for murine studies of Tregs and T1D progression (Anderson and Bluestone, 2005; Atkinson and Leiter, 1999; Delovitch and Singh, 1997). Susceptibility to diabetes correlates with the loss of FoxP3-expressing Tregs (Thornton and Shevach, 1998; Baecher-Allan and Hafler, 2006). T1D can be prevented by adoptive transfer of freshly isolated polyclonal and antigen-specific Tregs (Szanya et al., 2002; Tarbell et al., 2007; Tang et al., 2004; Lepault and Gagnerault, 2000; Lundsgaard et al., 2005). This argues strongly that Tregs play a crucial role in the pathogenesis, and potentially the treatment, of T1D. In NOD mice lacking Tregs, 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 Tregs 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).

While adoptive Treg therapy has been primarily focused on T1D and HSCT, there are numerous other diseases in which Treg therapy is worthy of consideration. In the aforementioned study by Sakaguchi and colleagues, mice depleted of Tregs developed anti-dsDNA Abs reminiscent of SLE (Sakaguchi et al., 1995). Others have noticed that SLE patients have fewer Tregs than healthy individuals, that such Tregs are more susceptible to Fas-mediated apoptosis, and that Tregs from patients with active disease have impaired activity (Crispin et al., 2003; Liu et al., 2004; Miyara et al., 2005). Notably, ex vivo expansion of Tregs from SLE patients corrected their functional defect (Valencia et al., 2007), suggesting that ex vivo expanded, autologous Tregs might have a beneficial effect in SLE patients. Similarly, patients with Sjögren's syndrome have reduced levels of Tregs in their peripheral blood. Furthermore, their salivary glands, a common autoimmune target in this population, contain markedly reduced numbers of Tregs (Li et al., 2007; Liu et al., 2008). Importantly, on a per-cell basis, Tregs from Sjögren's syndrome patients and Tregs from age-matched healthy controls functioned equivalently, suggesting that Treg 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 therapy is less clear. Results from murine models of MS (experimental autoimmune encephalomyelitis, EAE) question the benefit of adoptive Treg transfer. If polyclonal or antigen-specific Tregs are infused prior to acquisition of disease, EAE can be prevented. However, if the Tregs are infused after disease initiation, their therapeutic value is considerably diminished (Van de Keere and Tonegawa, 1998; Olivares-Villagomez et al., 1998). Examination of the ability of antigen-specific Tregs to suppress Tconvs isolated from the CNS of animals with active EAE revealed that Tconvs were highly resistant to the anti-proliferative effects of antigen-specific Tregs, perhaps due to the fact that these Tconvs secrete high levels of IL-6 and TGF-β (Korn et al., 2007). There is evidence that Tregs function poorly in inflammatory environments (Lewis et al., 2008).

Since most, if not all, MS patients present with active disease, 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 Tregs may be of benefit. Additionally, several studies have demonstrated that Tregs isolated from MS patients have diminished suppressive activity (Haas et al., 2005; Huan 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, Tregs will provide therapeutic benefit. Preclinical studies to determine whether Tregs derived from progenitor cells such as gene corrected, 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 infusional therapy in alloimmunity

In cyclosporin-treated rats with long-term cardiac allograft survival, adoptive transfer of $CD4^+25^+$ T cells resulted in tolerance (Hall et al., 1990). The demonstration that Tregs from naïve mice prevented rejection of allogeneic skin grafts in nude mice given $CD25^-$ Tconvs (Sakaguchi et al., 1995) further set the stage for the application of Tregs to solid organ transplant settings. In vitro studies in which positive costimulatory pathway blockade inhibited alloantigen-specific responsiveness demonstrated that Tregs 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 Tregs contained in the transfusion product (Jarvinen et al., 2003). A series of preclinical rodent studies demonstrated that Tregs 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 $CD4^+25^+$ or $CD4^+45RB^{lo}$ Tregs suppressed pancreatic islet allograft rejection (Sanchez-Fueyo et al., 2002; Davies et al., 1999; Hara et al., 2001).

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 Tregs was highly effective in preventing acute or chronic GVHD (Taylor et al., 2002; Zhao et al., 2008). Further, in vivo activated donor or host Tregs 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 anti-donor alloreactive Tconvs could reject donor bone marrow, which was preventable by donor Treg infusion (Joffre et al., 2004; Hanash and Levy, 2005; Taylor et al., 2004). Donor Treg 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 Tregs, 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 Tregs.

Challenges and clinical applications of human Treg large-scale manufacturing

Human Tregs have no unique cell surface markers. Translating Tregs into the clinic has been hindered by the relatively high frequency of activated Tconvs present in the $CD4^+25^{++}$ fraction and the limited availability of good manufacturing practice (GMP)-compatible procedures for removing these contaminants. Furthermore, the relatively low frequency of Tregs in human peripheral blood, combined with data from mouse models indicating that large doses of polyclonally activated Tregs are required to suppress GVHD, indicate that for most applications, Tregs will require ex vivo expansion. Expansion approaches that provide strong TCR signals tend to preferentially expand Tconv at the expense of Tregs. Thus, it is essential to either initiate the expansion culture with highly purified Tregs or create culture conditions that favor Treg outgrowth. Most preclinical human data analyzing human Tregs have been generated using 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 isolation under GMP conditions due to the unavailability of equivalent GMP reagents and lack 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, as assays performed on Tconv populations can be directly translated to Tregs. Likewise, tracking measures employed to establish Tconv identity, such as HLA verification and barcoding, require no adaptation for use in Tregs. Demonstrations of Treg purity and potency are more problematic. Foxp3 expression is a good surrogate for Treg purity, but given current expansion techniques, it is unlikely that Treg cultures will be 100% $Foxp3^+$ following expansion. It will be important to determine “acceptable” levels of contamination, from both quantitative and qualitative perspectives. Acceptable levels may be disease-dependent. For reasonably well-controlled autoimmune diseases such as T1D the acceptable level of non-Foxp3 expressing cells will be lower than the acceptable level of non-Foxp3 expressing cells in acute GVHD therapy. From the qualitative perspective, it is possible, for example, that contaminating naïve Tconvs will be less problematic in autoimmune disease applications since it is unlikely that the pathogenic Tconvs are in this subset, while contaminating memory cells may be less problematic in GVHD or organ transplant situations, since the alloreactive response is likely to be contained in the naïve 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.

The potency issue clearly separates Tregs from other T cell-based therapies. For Tconv therapy there are *in vitro* surrogates, such as cytokine secretion by CD4 T cells and lytic potential of $CD8^+$ T cells, that correlate well with in vivo efficacy. Unfortunately, our understanding of Treg function lags considerably behind our understanding of Tconv function. Multiple mechanisms have been implicated in Treg function but there is no clear understanding of how suppression occurs in vivo (Tang and Bluestone, 2008). Unfortunately, the correlation between *in vivo* Treg activity and activity measured by the popular *in vitro* CFSE dilution is not perfect. We recently reported that $CD4^+$ Tconvs expanded in the presence of rapamycin were highly effective in an *in vitro* suppression assays, 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 Tregs 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 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 T regs to prevent anti-human immune responses, but these new techniques are not yet widely used. 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 Tregs can also be used with non-human primates (Haanstra et al., 2008; Ansari et al., 2007; Anderson et al., 2008). Given that non-human primates have been instrumental in improving and validating organ transplant protocols (Lechler et al., 2005), it would seem likely that transplant studies in non-human primates would guide the in vivo use of human Tregs. However, it is important to note that non-human primate models were used in the evaluation of the superagonistic anti-CD28 Ab TGN1412 and no toxicities were observed, questioning the utility of non-human primate models for Treg therapy (Schraven and Kalinke, 2008). Understanding when non-human primate studies will be informative and appropriate will drive the clinical translation of ex vivo expanded Tregs. In any case, during the developmental stages of Treg therapy, there is an urgent need for *in vivo*-validated *in vitro* Treg functional assays so that the quality of expanded Treg cultures can be accurately assessed.

Current Phase I clinical trials evaluating adoptively transferred Tregs

Allogeneic Tregs 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 using a sub-saturating concentration of anti-CD25 antibody to capture the $CD25^{Bright}$ fraction (Godfrey et al., 2005; Godfrey et al., 2004). The final isolation products contained ~50% Tregs as assessed by CD4/CD25/FoxP3 flow cytometry. Further enrichment based upon FoxP3 expression levels is not possible since FoxP3 detection requires permeabilization. By eliminating the ex vivo expansion step, Edinger et al. have initiated a trial in which freshly isolated allogeneic donor Tregs are infused, avoiding the preferential expansion of contaminating Tconvs. These fresh Tregs 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 co-authors and their colleagues, umbilical cord blood (UCB) was used for Treg isolation since cord blood is virtually devoid of memory T cells. In ongoing trials, non-myeloablated or myeloablated recipients of two unrelated cord blood units used for hematopoietic reconstitution, given with standard of care GVHD prophylactic agents (cyclosporine A; mycophenolate mofetil), also received third-party, HLA partially-matched

Tregs. Although $CD25^{bright}$ Tregs are more readily purified from umbilical cord blood than peripheral blood (Godfrey et al., 2005; Godfrey et al., 2004), only ~5−7.5 $\times 10^6$ Tregs can be isolated from a frozen UCB unit. Thus, to achieve high Treg:Tconv ratios, cord blood Tregs used in this study were expanded using anti-CD3/28-coated microbeads IL-2, and rapamycin, resulting in ~200–300 fold expansion in ≤ 3 weeks. The ability to expand peripheral blood Tregs was advanced by the discovery that the immunosuppressant rapamycin selectively expands or preferentially preserves Tregs over Tconvs (Battaglia et al., 2005) due to the FoxP3 induced expression of Pim2, a serine/threonine kinase that confers rapamycin resistance (Basu et al., 2008). Although rapamycin typically decreases overall Treg expansion ~10-fold, it is required in order to consistently generate functional Tregs expanded from enriched but not pure Tregs (Golovina et al., 2008).

As noted above, cord blood Tregs are an attractive therapeutic modality. They are largely naïve, they have long telomeres, and they are easily separated from Tconvs due to the reduced complexity of cord blood T cell subsets. One drawback of cord blood is that to date it has been employed as an allogeneic cell source, necessitating HLA matching and opening the possibility of host v. graft or graft v. host disease. However, within the last decade or so, banking of infant cord blood has increased markedly, so it is possible to envision autologous cord blood therapy and in particular, therapy with ex vivo expanded autologous cord blood-derived Tregs. Investigators at the University of Florida (Haller, Atkinson and Schatz) have infused 23 T1D individuals with autologous, unfractionated cord blood. Since cord blood contains mesenchymal stem cells, Tregs 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 cord blood to treat autoimmune disease, and it opens many exciting possibilities for future trials. As was mentioned above, it is possible to envision future approaches with similar advantages of cord blood using autologous Tregs derived from induced pluripotent stem cells.

Potential dangers of human Tregs therapy

Like all therapies, clinical use of ex vivo expanded Tregs is associated with potential risks. Perhaps the most troublesome is the possibility of expanded Tregs reverting to Tconvs, especially if antigen-specific Tregs are infused. The use of $Foxp3^{EGFP}$ knockin mice has been instrumental in understanding Treg plasticity (Wang et al., 2008; Fontenot et al., 2005). TGF-β is required for both Treg and Th17 differentiation, with the ultimate fate decision resting on interactions between SMAD4, RORγ, RORα, STAT3, IL-1 and IL-6 (Yang et al., 2008; Radhakrishnan et al., 2008). The majority of adoptively transferred Tregs maintain their suppressive activity, but a minority of cells lose Foxp3 expression and can differentiate into Tconvs (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 therapy. Since Foxp3 is the master regulator of Treg function, alterations in Foxp3 expression or activity are likely involved in converting Tregs to Tconvs. 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 function and fidelity in vivo.

There are also concerns that excessive Treg 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 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 Tregs restrict protective immune responses. The use of suicide vectors may be an attractive way to eliminate introduced Tregs if excessive immunosuppression is observed. However, this would

likely require some form of gene therapy, which requires its own careful assessment of risks and rewards.

Future directions for Tregs in the clinic

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

After antigen encounter, a subset of naïve Tconvs become memory T cells. Do memory Tregs 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 co-expressing CD4, CD25, CD45RA, and Foxp3 declines with age in healthy controls; the decline is more severe in MS patients (Haas et al., 2007). While this suggests that maintaining a pool of naïve Tregs is important, it is important to note that the existence of Treg memory cells has not been demonstrated. Since Tregs 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 subsets can be defined phenotypically. Both $CD45RA^{+}$- and $CD45RO^{+}$- $CD25^{++}$ Tregs exist, but it is not clear whether the CD45RO cells represent contaminating Tconv cells. In any case, $CD45RO^{+}CD25^{++}$ cells exhibited significantly less suppressive activity post-expansion than $CD45RA^{+}CD25^{++}$ cells (Hoffmann et al., 2006), suggesting that these cells do not have the properties of memory Tregs

As evident from our title, it is unclear what constitutes a therapeutic dose of Tregs. The answer depends on many factors, including the specific disease targeted and whether polyclonal or antigen-specific Tregs are employed. Defining Treg dosing strategies requires large-scale Treg expansion capacity. It has been reported that using a murine cell-based artificial APC (aAPC) system, human Tregs 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 function, so the purity and potency of these Tregs is difficult to ascertain. Subsequent studies have reported more modest levels of expansion using anti-CD3/CD28 coated beads (of which GMP versions are available) in the presence of high levels of IL-2 (Earle et al., 2005; Godfrey et al., 2004). Inclusion of rapamycin results in a further decline in Treg yield. We have found that anti-CD3-Ab loaded K562-based aAPCs expand Tregs more efficiently, and the expanded cells exhibit greater purity and potency than cells expanded using anti-CD3/28 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 levels comparable to those used in $CD4^{+}$ Tconv adoptive transfer applications. Another advantage of K562-based aAPCs is that additional cell surface (costimulatory molecules) and secreted molecules (cytokines and chemokines) can be easily added to further refine human Treg expansion (Suhoski et al., 2007). To date, our data using human isolated from peripheral blood indicate that addition of costimulatory ligands other than

CD86 favors the expansion of contaminating Tconvs at the expense of Tregs (Golovina et al., 2008). In contrast, expansion of UCB Tregs is enhanced by the presence of additional costimulatory ligands {Hippen, 2008 275 /id}. This discrepancy is most likely a consequence of the substantially higher purity of the starting Treg populations from UCB. Lastly, 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 expansion using irradiated K562-based aAPCs.

The ability to overcome some of the limitations of Treg therapy by genetically reprogramming Tregs 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 re-direct polyclonal Tregs to specific targets. In murine models of T1D, antigen-specific Tregs are far more potent than polyclonal Tregs on a per-cell basis (Tang et al., 2004). However, current technology does not easily permit the identification of islet-specific Tregs in humans. Moreover, multiple rounds of expansion would likely be required to obtain therapeutic levels of Tregs, which might compromise their engraftment, persistence or function.. In murine models, introduction of a chimeric immune receptor (CIR) into Tregs 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; Li et al., 2005) could also be applied to improve TCR-transduced Treg therapy (Hori et al., 2002). We recently demonstrated that high affinity TCRs conferred greater effector function to Tconvs (Varela-Rohena et al., 2008). It will be interesting to determine whether this applies to Tregs as well. Additional gene modifications can be envisioned that may enhance the therapeutic potential of Tregs. 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 (Urnov et al., 2005) or lentiviral vector-mediated introduction of the wild type Foxp3 gene under the control of a Treg-specific promoter. Likewise, to prevent the Treg/Tconv conversion, Tregs could be engineered to express either Foxp3 so that these infused Tregs are less likely to convert to T conv. Lastly, as our knowledge of Treg immune suppressive mechanisms increases, additional opportunities to enhance Treg function via gene therapy will present themselves.

Since natural Tregs are present at a low frequency in peripheral blood, CD25-based purification techniques remove the vast majority of $CD4^+$ T cells. Because $CD4^+25^-$ T cells can be induced to express high FoxP3 levels, an alternative approach to natural Treg purification is to generate inducible Tregs (iTregs) by subjecting $CD4^+25^-$ T cells to conditions that result in the gain of suppressor cell function. Although some species-specific differences are likely to exist, murine iTregs can be generated following ex vivo exposure of naïve $CD4^+25^-$ or $CD4^+CDRO^-$ T cells to TGFβ (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 generation may include exposing $CD4^+25^-$ T cells to subimmunogenic DCs or to histone deacetylase inhibitors that specifically regulate FoxP3 expression. While potentially promising, there is a paucity of data regarding the yield, stability and plasticity of iTregs as assessed by both in vitro and in vivo measures. Thus, it would be premature to gauge whether iTreg generation may circumvent the technical limitations of producing high numbers of natural Tregs using 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 Tregs offer the ability to be highly specific and effective. Also, Treg 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 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 Tregs and provide better options for those suffering from autoimmune and other immune mediate disease states? Only time and hard work will answer this question but nonetheless one day, as our title implies, Treg therapy will be as commonplace as taking two aspirins.

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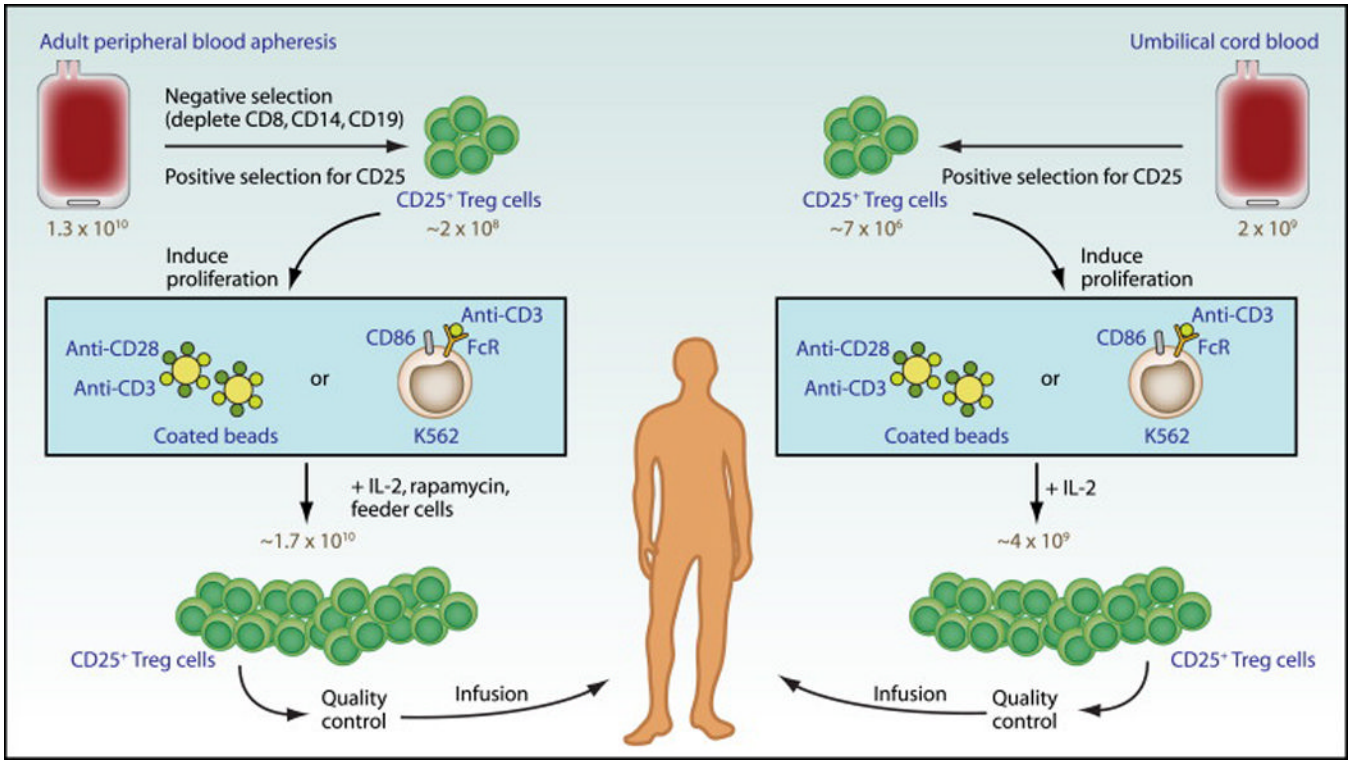

Figure 1. Clinical Applications of Human Treg Cells in Allo-HSCT

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.