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Colloquium

Therapeutic vaccination using CD4^{+}CD25^{+} antigen-specific regulatory T cells

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Autoimmune disease results from the dysregulation of basic tolerogenic processes designed to control self/non-self-discrimination. Approaches to treat autoimmunity have focused historically on potent immunosuppressives that block the activation and expansion of antigen-specific T cells before they differentiate into pathogenic T cell responses. These therapies are very efficient in reducing clonal expansion and altering early signaling pathways. However, once the pathogenic responses are established (i.e., autoimmunity), the interventions are less effective on activated and differentiated T cell subsets (including memory T cells) or acting in the presence of an inflammatory milieu to abort immune responses at the target tissue and systemically. Moreover, the current immunotherapies require continuous use because they do not redirect the immune system to a state of tolerance. The continuous treatment leads to long-term toxicities and can profoundly suppress protective immune responses targeted at viruses, bacteria, and other pathogens. Over the past decade, there have been tremendous advances in our understanding of the basic processes that control immune tolerance. Among the most exciting has been the identification of a professional regulatory T cell subset that has shown enormous potential in suppressing pathologic immune responses in autoimmune diseases, transplantation, and graft vs. host disease. In this review, we summarize current efforts to induce and maintain tolerance in the autoimmune diabetes setting by using therapeutic vaccination with CD4^{+}CD25^{+} regulatory T cells. Emphasis will be placed on approaches to exploit regulatory T cells either directly or through the use of anti-CD3 immunotherapy.

Regulation of the immune response to self-antigens is a complex process that depends on maintaining self-tolerance while retaining the capacity to mount a robust immune response. T cells specific for these autoantigens are present in most normal individuals but are kept under control by multiple diverse peripheral tolerance mechanisms. For at least 30 years, there has been the notion that in addition to T cells that mediate effector immune responses to combat infections and mediate graft rejection, there are classes of regulatory/suppressor T cells that exist to control immunity (reviewed in ref. 1). Early on, CD8^{+} T cells were identified that suppress immune responses through their direct cytotoxicity on antigen-bearing cells or through cryptic suppressor factors that were poorly characterized (2). However, during this early period, there were already hints that the quintessential helper T cells subset, CD4^{+} T cells, also may have regulatory activity. North and Awwad (3) showed that depletion of CD4^{+} T cells by using anti-CD4 mAbs resulted in tumor rejection. This modern view of CD4^{+} regulatory T cells (Tregs) was enhanced by the observations by Sakaguchi *et al.* (4, 5) that the adoptive transfer of T cells depleted of CD4^{+}CD25^{+} cells induced multiorgan autoimmunity in the recipient animals. These studies complemented ongoing efforts by a number of groups in England and France who demonstrated antigen-specific Treg populations in mice and rats (6–9). In fact, multiple investigators provided compelling data to support the existence of Tregs in rodents, especially in those animals that had undergone certain immunotherapeutic interventions in the allogeneic transplant or autoimmune setting. For example, populations of CD4^{+} peripheral T cells and thymocytes were shown to prevent induction of autoimmune thyroiditis in an antigen-specific manner (10). The Tregs were shown to be driven by peripheral autoantigen and could be extracted from mice maintaining long-term allografts. Most importantly, Gershon and Kondo (11), and subsequently Cobbold and Waldmann (8), developed the concept of infectious tolerance, where cells from tolerant animals could be transferred to naïve recipients suppressing not only the original antigen specificities but other antigens linked through the same antigen-presenting cells. However, progress in this area was slow and tedious, often fraught with skepticism in the community because of difficulty in defining the precise phenotype of these cells, their antigen specificity, and the mechanistic basis for the suppressive activity.

A New Age for Suppressor T Cells

In the last few years, there has been a rebirth of suppressor cells as one of the most central mechanisms of immune regulation. Investigators have found that, in most instances, these CD4^{+} T cells express CD25, GITR, CTLA-4, and CD62L (1, 5, 12, 13). This minor CD4^{+} T cell subset was shown to develop in either the thymus or the periphery to maintain the homeostatic equilibrium of immunity and tolerance. Significantly, a specific transcription factor, forkhead box p3 (Foxp3), has been identified (14) that controls Treg development and expression of the suppressive phenotype. There are emerging data in humans that suggest that animal models of Tregs are indeed relevant to human disease. A human multiorgan autoimmune disease has been observed in patients deficient in the Foxp3 transcription factor (15). Regulatory function has been demonstrated in coculture experiments with CD4^{+} T cells expressing high levels of CD25 (16) and has been shown to be deficient in patients with type 1 diabetes (T1D), multiple sclerosis, and other immunological diseases (17–19). Finally, Tregs are induced under certain therapeutic interventions, including treatment with anti-CD3 antibodies in early-onset diabetics and islet transplantation as well as certain altered antigenic stimuli (20–22). These cells have immunosuppressive properties similar to preexisting CD4^{+}CD25^{+} T cells and may be influencing the long-term effects of these therapies.

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Abbreviations: Treg, regulatory T cell; TGFβ, transforming growth factor β; T1D, type 1 diabetes; NOD, nonobese diabetic; FcR, Fc receptor; TCR, T cell receptor.

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Growing data suggest that there are likely distinct subsets of Tregs. In many experimental systems, Tregs seem to function by the production of immunosuppressive cytokines, particularly transforming growth factor β (TGFβ) and IL-10 (23, 24). Other studies indicate that suppressive function requires cell–cell contact and cannot be attributed to cytokine-mediated suppression (12). The finding that Tregs express a membrane form of TGFβ that is responsible for their suppressive activity (25) and our own work suggesting that Tregs are involved in regulating diabetes (26) support a critical role for these suppressive cytokines in the bystander regulation observed in this setting. In this regard, it has been suggested that Tregs can be delineated into two subsets: natural and adaptive Tregs (27). Natural Tregs develop during the normal process of T cell maturation in the thymus, resulting in an endogenous, or natural, population of antigen-specific Tregs that survives as a long-lived population in the periphery poised to prevent potentially pathological autoimmune reactions. In most settings, these cells constitutively express CD25, CTLA-4, GITR, and CD62L and represent 5–10% of $CD4^+$ T cells. The second subset of Tregs, so-called adaptive Tregs, develops as a consequence of activation of mature T cells under particular conditions of suboptimal antigen exposure and/or costimulation. The adaptive subset of Tregs functions mainly as a homeostatic control over various adaptive immune responses. These cells, termed Th3 or Tr1 cells by some investigators, do not always express the quintessential cell-surface markers. Whereas *in vitro* suppression by natural Tregs is independent of known immunosuppressive cytokines and depends on cell–cell contact by an unknown mechanism, adaptive Tregs produce immunosuppressive cytokines such as TGFβ and IL-10 to mediate suppressive activity. Thus, it is now evident that the opportunities to target Tregs for the treatment of autoimmunity, graft vs. host disease, and organ transplantation have added a new weapon to the immunotherapy arsenal. The remainder of this review will focus on several aspects of Tregs in T1D. What is the role of CD28 costimulation in their development and survival? How can these cells be manipulated *in vivo*? What is the potential to use these cells, expanded *ex vivo*, as a therapeutic vaccine?

Tregs and T1D

The nonobese diabetic (NOD) mouse model of autoimmune diabetes was developed some 25 years ago (28), having been identified by an inappropriate response against self-proteins resulting in T cell-mediated destruction of the pancreatic islets as well as other tissues. The disease results from a progressive activation of $CD4^+$ and $CD8^+$ T cells that recognize islet antigens such as insulin, proinsulin, glutamic acid decarboxylase, the protein phosphatase I-A2, and heat shock protein 60 (1). The development of the disease is B cell-dependent (29), but the autoantibodies resident in diseased mice are not pathogenic (30). Most significantly, the autoimmune disease is highly regulated. There is a significant delay between the first signs of autoreactive T cell activity (at 3–4 weeks of age when insulitis appears) and typical onset of significant islet cell destruction and diabetes (up to 20 weeks of age). In the mid 1990s, Chatenoud and colleagues (31) demonstrated that a population of $CD4^+CD62L^+$ T cells from young NOD mice was able to protect mice in a cotransfer experiment with pathogenic cells from a diabetic animal. Similar studies were performed by using a combination of CD25 and CD62L because this combination appeared to best define the Treg subset. We observed that $CD4^+CD62L^+CD25^+$ Tregs constitute about 5% of the circulating $CD4^+$ T cells in NOD mice (32). Although this number is significantly lower than that observed in other strains, NOD Tregs are functionally competent (especially early in life). Moreover, depletion of $CD25^+$ cells from young NOD results in the development of diabetes. Chatenoud and colleagues (26) demonstrated that the protection by these cells depended on TGFβ, because treatment with two different anti-TGFβ mAbs at the time of Treg transfer reversed the protection. More recently, we have observed that NOD Tregs can be directly transferred into lymphosufficient animals devoid of Tregs, thus preventing the exacerbated autoimmunity observed in this setting (32).

Role of CD28/B7 Pathway in Homeostasis of Treg Cells

The CD28/B7 pathway has been considered the quintessential T cell costimulatory molecules involved in initiating T cell responses. However, early studies of the role of CD28 costimulation in the spontaneous autoimmune T cell-mediated diabetes of NOD mice yielded a surprising result. CD28KO and B7-1/B7-2KO NOD mice developed diabetes earlier and with a higher incidence as compared with their littermate controls (13). Similar findings were obtained in NOD mice treated with the CD28 antagonist, murine CTLA-4Ig, at 2–4 weeks of age, corresponding to the first visible signs of the autoreactive process (13). Thus, we hypothesized that CD28/B7 interactions may play a critical role in Treg development and function in NOD mice. Examination of NOD mice in which the CD28/B7 pathway was disrupted demonstrated a profound decrease in the number of peripheral $CD4^+CD25^+$ T cells. CD28KO mice showed a clear reduction of $CD4^+CD25^+$ cells in the thymus, suggesting a crucial role for this pathway in Treg development. Moreover, the CD28/B7 pathway was critical in the peripheral homeostasis of these cells. Normal mice injected with the CD28 antagonist, CTLA-4Ig, or with a combination of anti-B7-1 and anti-B7-2 mAbs demonstrated a 60–80% reduction in $CD4^+CD25^+$ cells in the periphery. This was not simply a result of a loss of the CD25 expression as a marker for these cells, because similar results were obtained using carboxyfluorescein diacetate succinimidyl ester labeling to track the cells *in vivo*. In addition, CD28/B7 blockade in thymectomized wild-type mice resulted in a similar reduction in Tregs consistent with a direct role for CD28 in peripheral Treg homeostasis. In fact, we have demonstrated that CD28 costimulation in Tregs is critical to their expansion and survival *in vivo* (33). Finally, diabetes could be reversed in NOD mice by reconstitution of CD28KO mice with $CD4^+CD25^+$ T cell population from wild-type NOD mice (32). These data suggest an important role for CD28 in both the development and maintenance of the Treg subset.

The importance of this work is severalfold. First, these data must be seen in light of ongoing efforts to develop drugs targeted at this critical costimulation pathway. CTLA-4Ig and a high-affinity mutant form, LEA29Y, are currently in clinical trials in organ transplantation and autoimmunity (34). The goal of blocking CD28 costimulation as a means of inhibiting T cell activation and early clonal expansion is justified; however, care must be taken to examine the effects of these drugs on Treg populations, especially in the autoimmune setting where effector cells are already established and less costimulation-dependent. In this regard, recent work from our group suggests that engagement of either CD28 ligand B7-1 (CD80) or B7-2 (CD86) is sufficient to promote Treg development and survival (H. Jordan and J.A.B., unpublished observations). In sharp contrast, CD28/B7-2 is the dominant costimulatory pathway for the development of pathogenic responses in NOD diabetes. Thus, it is possible that selective inhibition of CD28/B7-2 pathway interactions may compromise pathogenic T cell responses while maintaining Treg activity. The second important implication of the observations concerning CD28 and Tregs is its potential use in expanding this population. Previous studies have suggested that Tregs are a largely anergic population unable to expand efficiently *in vitro* (12, 35). This anergic phenotype of Tregs is reversed in the presence of anti-CD28 mAb-mediated costimulation (12). As we describe below, we have taken advantage of this understanding

of the critical role of CD28 to develop a robust approach to expanding this regulatory subset (35).

Induction of Treg Activity by Anti-CD3ε mAb Therapy in NOD Mice and Patients with T1D

Many therapies that reduce clonal expansion in an antigen-specific manner (glutamic acid decarboxylase, insulin, etc.) have profound effects during the prediabetic stage of disease (36). This outcome is because of their selective effects on naïve antigen-specific cells before the occurrence of epitope spreading, which significantly expands the antigenic repertoire. By contrast, less than half a dozen therapies have been shown to reverse diabetes once the clinical manifestations of the disease are evident. Moreover, drugs like cyclosporin, which reverses diabetes in mice as well as humans, do not have a durable effect because, although these drugs clear the immune infiltrate in the attacked tissues, once the therapy is discontinued the infiltrates return with increased pathogenic activity and virulence (37). Thus, long-term, durable tolerance depends on the redirection of the immune system to a tolerant state exemplified by a combination of antigen-specific immune regulation and altered cytokine balance that actively protects the target tissue from destruction.

Anti-CD3 mAbs are potent immunosuppressive agents that have been approved for use in patients undergoing acute kidney allograft rejection. The efficacy of the mAb suggested that it could be effective in a number of other immune disorders such as autoimmunity. In fact, the anti-CD3 mAbs have been shown to have unique tolerogenic properties (38, 39). Treatment of NOD mice presenting with new-onset diabetes with low doses of anti-CD3 mAbs induced a return to permanent normoglycemia and durable disease remission in 80% of mice. This therapy restored self-tolerance because anti-CD3 antibody-treated NOD mice, unlike control untreated overtly diabetic NOD females, did not destroy syngeneic islet grafts but were fully responsive to other unrelated allografts. Unfortunately, the original mouse anti-human CD3 mAb, OKT3, could not be used in patients presenting with autoimmune diseases because of severe toxicities associated with an activation-induced cytokine storm (40). Thus, in an effort to eliminate the toxicity that occurred with OKT3, we developed a humanized OKT3 and substituted alanines for leucines at positions 234 and 235 to abolish the binding of the IgG1 molecule to Fc receptors (FcRs) (41, 42). Initial studies with this drug indicated that the mutation prevented multivalent binding, resulting in a dramatically reduced activation profile both *in vitro* and *in vivo* (41, 42). Moreover, the mutated bivalent mAb was equivalent in its ability to bind and modulate T cell activity. In a preclinical study, short-term immunotherapy of overtly diabetic NOD mice, with an FcR nonbinding anti-mouse CD3 mAb, restored durable self-tolerance to autoantigens and prevented autoimmune recurrence in syngeneic islet grafts (38, 39). More importantly, in a randomized controlled phase I/II trial of the FcR nonbinding anti-CD3 mAb, hOKT3γ 1(Ala-Ala), in newly diagnosed patients with T1D, a single 14-day course decreased the rate of loss of insulin production for >1 year and improved glycemic control with concomitant reduction in insulin use. This was achieved without the need for continuous immune suppression and persisted at a time when T cells were quantitatively normal (20).

Parallel efforts have continued to better define the immunologic activity of the FcR nonbinding anti-CD3 mAb. We observed that treatment of mice with the FcR nonbinding anti-CD3 mAb led to a dramatic decrease of pathogenic cells short term in the inflamed tissue due to apoptosis of the infiltrating cells. This short-term clonal deletion was followed by a wave of protective T cells entering the pancreas, characterized by an increase in IL-10- and TGFβ-producing Tregs, especially in the draining pancreatic lymph nodes of treated animals. Similar changes were noted in patients treated with the hOKT3γ1 (Ala–Ala). In those studies, at the conclusion of drug treatment, a subpopulation of CD4$^+$CD25$^+$ Tregs was observed in the blood that was producing IL-10 but not IFN-γ (20). Similar results were observed in human islet transplant recipients (21). In the latter study, the cells could be isolated from the treated individuals and shown *in vitro* to suppress alloantigen-specific responses. One unanticipated observation was that the anti-CD3 treatment was equally capable of reversing diabetes in the CD28-deficient NOD mice, which, as discussed above, lack Tregs. Further analysis of these animals revealed that the antibody therapy appears to induce Foxp3$^+$ Tregs *de novo* (M. Belghith, J.A.B., and L. Chatenoud, unpublished observations). These results suggest that the Tregs induced in wild-type NOD mice also may be induced *de novo*. This hypothesis fits with the observation that the Treg activity after anti-CD3 treatment can be reversed by blocking TGFβ, consistent with the notion that the therapy differentiated naïve uncommitted T cells into Tregs (26). The precise mechanism of how peripheral engagement of T cells with certain stimuli such as the FcR nonbinding anti-CD3 mAb leads to Treg development and expansion is unknown. However, recent studies have shown that the same antigen expressed in different sites generates CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ Tregs (43). In addition, presentation of antigenic peptide by using a slow-releasing pump also leads to Treg development (22). Together, these studies support the concept that either quantitative or qualitative differences in T cell receptor (TCR) stimulation result in distinct differentiation signals, which in some cases promote Treg rather than T effector cell development.

Expansion of Tregs and Treg-Based Therapy for T1D

The use of somatic cell therapy as an adjunct to tolerance induction has gained increasing interest because of the unique modulating activities of Treg subsets. However, the field has been limited by a combination of an inability to define antigenic specificities of Tregs, small numbers of circulating Tregs, and an inability to expand a functional Treg population *in vitro*. Thus, until now, it has been impossible to harness the potent suppressive activity of Tregs for the treatment of autoimmune disease and transplant rejection. To address these problems, we developed *in vitro* methods for clonally expanding antigen-specific CD4$^+$CD25$^+$ Tregs. Previous studies had suggested that Tregs were anergic; that is, they would not proliferate effectively by using conventional stimulatory approaches (44, 45). This hypothesis is in sharp contrast to recent observations that Tregs proliferate quite actively *in vivo* in an antigen- and costimulation-dependent manner (35, 46). However, as noted above, Tregs are particularly sensitive to both TCR and CD28 signals. Moreover, gene array analyses showed that Tregs express high levels of growth inhibitor genes, including *Suppressor of Cytokine Signaling-1* and *2*, *CTLA-4*, *PD-1*, and *TGIF*. Thus, we hypothesized that overcoming this cell-cycling block would require a combined strong TCR/CD28 signal plus high-dose IL-2. The protocol that has been most successful has combined anti-CD3- and anti-CD28-coated beads in the presence of 1,000–2,000 units of recombinant IL-2. Under these conditions, sorted CD4$^+$CD62L$^+$CD25$^+$ Tregs expanded as much as 200- to 250-fold within 14 days. The expanded CD4$^+$CD62L$^+$CD25$^+$ cells maintained a Treg phenotype as they continued to express high levels of cell-surface molecules (CD25, CD62L, PD-1, CTLA-4, and TRAIL). Most importantly, the expanded cells expressed high levels of Foxp3. Similar studies have been performed by using human sorted CD4$^+$CD25$^+$ Tregs. Sorted CD4$^+$CD25^{high} human T cells from peripheral blood could be expanded by at least 100-fold. These cells were effective in suppressing *in vitro* proliferative responses to either anti-CD3 or allogeneic stimuli. In both cases, the suppression *in vitro* of proliferation and

cytokine production was equal or greater than freshly isolated Tregs. The suppressor activity depended on TCR engagement and cell–cell contact. However, although the expanded cells produced both IL-10 and TGFβ, these cytokines were not involved in the *in vitro* suppression.

We next examined the ability of the expanded Tregs to suppress diabetes *in vivo* in NOD mice. As previously shown for freshly isolated Tregs, the $CD4^+CD62L^+CD25^+$ cells could suppress the development of diabetes in an adoptive transfer system where the cells were coinjected with diabetogenic NOD cells into a lymphopenic host. However, the suppression was relatively inefficient because as many as 10 million to 20 million cells were required to block diabetes induction by the polyclonal effector cells. These results were not unlike those observed for freshly isolated polyclonal NOD Tregs and most likely reflected the small precursor frequency of islet antigen-specific Tregs within the polyclonal population. This hypothesis was confirmed in a carboxyfluorescein succinimidyl ester tracking study. Polyclonal expanded NOD Tregs were labeled with the membrane dye and injected into normal prediabetic NOD recipients. At various time points the animals were killed, and the cells were examined for their antigen-specific proliferative capacity based on carboxyfluorescein succinimidyl ester dilution that occurs at each cell division. Although the expanded Tregs demonstrated normal homeostatic properties *in vivo* (homing, proliferation, and survival), $<1\%$ of the cells were observed to respond and proliferate in the draining pancreatic lymph nodes. In fact, the proliferation was no different in the pancreatic lymph nodes that are the sites of islet antigen exposure than in other distal lymph nodes (such as the inguinal lymph node) that do not present islet autoantigen. Therefore, effective Treg activity depends on both an appropriate phenotype and a high-frequency autoantigen specificity (32, 35). Thus, it would be hard to imagine how one could exploit polyclonal Tregs in an organ-specific autoimmune disease such as T1D, because the consequence of the adoptive transfer of these cells would be inefficient antigen-specific regulation with the possibility of pan-immunosuppression, resulting in increased susceptibility to infectious disease and cancer.

Next, a model was developed to specifically examine the expansion and function of antigen-specific Tregs in autoimmunity. $CD4^+CD25^+$ Tregs were isolated from the BDC2.5 TCR transgenic mouse strain (35). These mice, developed on the NOD background, express a TCRα and β chain derived from an islet antigen-specific T cell clone. T cells from these animals can transfer diabetes if activated *in vitro*. However, the BDC2.5 NOD mice do not develop diabetes unless bred onto a TCRα-knockout or RAG-knockout background. It has been hypothesized that the reason for this deficiency is that a population of Tregs exists in the wild-type BDC2.5 transgenic mice in part because of the use of alternative TCRα chains. Therefore, we examined these transgenic mice for the existence of Tregs. We observed that as many as 5% of the $CD4^+$ transgenic$^+$ T cells were $CD62L^+CD25^+$. Thus, we sorted this subset of cells, expanded them as above, and tested for their ability to suppress T effector cells both *in vivo* and *in vitro*. As with the polyclonal expanded Tregs, the BDC2.5 Tregs suppressed *in vitro* proliferation and cytokine production in assays stimulated by using anti-CD3 mAbs. However, in addition, the expanded BDC2.5 Tregs were able to suppress antigen-specific T effector cell responses in assays where the BDC2.5 Tregs were activated by a receptor-specific mimotope peptide. Most importantly, the expanded Tregs from the BDC2.5 TCR transgenic mice were highly efficient *in vivo* in suppressing diabetes in multiple model systems. Adoptive transfer of as few as 2×10^6 BDC Tregs prevented diabetes in which 1×10^7 T cells from diabetic NOD mice were transferred into NOD RAGKO mice. Moreover, the expanded Tregs restored normoglycemia in new-onset diabetic NODs as well as prolonged islet autograft in long-term diabetic NOD recipients. Finally, the expanded Tregs could reverse the Treg defect in CD28KO NOD mice such that as few as 150,000 Tregs fully prevented disease occurrence in these highly diabetes-prone, Treg-deficient animals.

We examined the selectivity of the Treg activity *in vivo*. The observation that the monoclonal antigen-specific BDC2.5 Tregs could suppress a polyclonal diabetic T effector population raised the possibility that the bystander effect of the Tregs might result in global immune suppression in treated animals. The effects of the auto islet-specific Tregs on rejection of allogeneic islet cells in diabetic NOD mice were tested. We observed that the islet-specific Tregs had no effect on alloimmunity. Moreover, we observed that the BDC2.5 Tregs only proliferated in the draining pancreatic lymph nodes and not other antigen-deficient tissues. Together, these results demonstrate that expanded antigen-specific Tregs are far more efficient at mediating immune regulation than polyclonal Tregs. The data demonstrate that although the Tregs may mediate bystander suppression in the effector phase, the effects are largely local, perhaps because of the short range of cytokine activity or limited effects only on antigen-presenting cells expressing multiple antigens.

In summary, it now appears that Tregs provide an essential and controllable arm of the immune system. Although the cells can be thymically derived, certain antigenic challenges or cytokine milieus promote Treg differentiation and expansion. Most importantly, small numbers of antigen-specific Tregs can reverse autoimmunity after disease onset, suggesting an approach to cellular immunotherapy for autoimmunity and transplantation. Thus, efforts to develop therapeutic vaccines for organ-specific autoimmunity using this type of cellular therapy will depend on identifying and selectively expanding autoantigen-specific Tregs in humans. One exception may be systemic diseases such as graft vs. host disease, where the precursor frequency of the alloantigen-specific Tregs within the polyclonal population has been shown to be sufficient to block disease (B. Salomon, Q.T., and J.A.B., unpublished observations). In any case, the hope is that these cells circulate in the peripheral blood of patients with active disease. Moreover, techniques will need to be developed that allow for their isolation and expansion either by using antigen presented on dendritic cells (47) or by using specific MHC-peptide agonists in the presence of adequate costimulation and growth factors. Once expanded, these highly active cells can be transferred into diseased individuals to reverse disease and promote long-term tolerance.

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