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Regulatory T Cell-based Therapies for Autoimmunity

Benjamine Arellano, Ph.D., **David J. Graber, Ph.D.**, and **Charles L. Sentman, Ph.D.**

The Center for Synthetic Immunity and the Department of Microbiology and Immunology, The Geisel School of Medicine at Dartmouth, One Medical Center Dr., Lebanon, NH 03756, USA.

Abstract

Autoimmune disorders are long-term diseases that adversely affect the quality of life for patients, and they are one of the top ten leading causes of death. While each autoimmune disorder is unique, they all are caused by a breakdown of tolerance against endogenous proteins. This leads to auto-inflammatory events that promote the destruction of organs in a humoral and cellular immune mediated manner. Treatment options for autoimmunity can involve the use of chemical and biologic agents that suppress inflammation. While these treatment options for patients have shown to be beneficial in autoimmunity, they can result in patients being vulnerable to opportunistic infections. Newer therapies aim to identify methods to specifically block auto-inflammatory immune cells while allowing for an intact immune response to other antigens. T regulatory (Treg) cells are a subtype of the adoptive immune cell that is capable of suppressing inflammatory events in an antigen-specific manner, but they are often poorly functioning within autoimmune patients. Treg cells have been well characterized for their immune modulating capabilities and preclinical and early clinical studies support their therapeutic potential for antigen-specific immune suppression. This review will examine the current understanding of Treg cell function and the therapeutic potential of enhancing Treg cells in patients with inflammatory disorders.

Autoimmune Diseases

Autoimmune diseases are estimated to affect between 3–5% of individuals in western societies (Cooper *et al.*, 2009). These diseases can be life-long diagnosis that adversely affect the health-related quality of life of patients and are one of the leading causes for death. It is unclear what triggers the initial event that breaks down tolerance to self-antigens and allows for the activation of auto-reactive immune cells (Goodnow *et al.*, 2005). There are positive associations between specific human leukocyte antigen (HLA) haplotypes and the presentation of specific autoantigens via the major histocompatibility complexes (MHCs) associated with these HLA haplotypes (Gough and Simmonds, 2007). The activation of the adaptive immune response against endogenous antigens allows for the targeting of disease specific organs (e.g., brain, kidney, liver) via antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (Hampe, 2012). Additionally, adoptive transfer of autoantigen specific T cells is capable of initiating disease in animal models,

Corresponding Author: Charles L. Sentman, Ph.D. (Charles.L.Sentman@dartmouth.edu).

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indicating that T lymphocytes also play key roles (Ohashi, 2002). While it is debatable which cell type plays a more important role in different autoimmune disorders, once these autoreactive T and B cells are activated, both can play key roles in the progression of the disease-specific pathology.

Therapies for autoimmune disorders aim to inhibit the proinflammatory immune response by depleting specific adaptive immune cell populations or inhibiting the activation of immune cells in target organs (Steinman *et al.*, 2012). While these strategies are helpful in limiting the proinflammatory immune response against endogenous proteins and tissues, they also inhibit protective immune responses and can leave patients immunocompromised and susceptible to various infections. Newer therapies are being designed to utilize the suppressive capabilities of regulatory T (Treg) cells to suppress autoimmune cells in an antigen-specific manner (von Boehmer and Daniel, 2013). Treg cells are diverse (Josefowicz *et al.*, 2012), being generated in the thymus [natural Tregs (nTregs)] or in the periphery [inducible Tregs (iTregs), also known as adaptive Tregs] via exposure to anti-inflammatory cytokines, such as TGF-β. Whether an nTreg or an iTreg, typically these regulatory T cells express forkhead box P3 (Foxp3), a transcription factor found to inhibit the expression of proinflammatory genes and upregulate the expression of antiinflammatory genes. Similar to proinflammatory T cells, Treg cells are activated via their T cell receptor (TCR) and the costimulatory molecule CD28. Because the specificity of the TCR against endogenous peptides is an important driver of the inflammatory response in autoimmune disorders, utilizing Treg cells that are specific for autoimmune peptides is a potentially valuable therapy because of the T cells potential to traffic to the site of inflammation and suppress the ongoing autoimmune response (Vandenbark and Offner, 2008). In this review, we will summarize the approaches and prospects for improving therapy for autoimmune diseases through manipulations of Treg cells.

Treg Mechanisms of Suppression in the Context of Autoimmunity

Treg cells have multiple suppressor mechanisms to mediate autoinflammatory events in patients. When activated, Treg cells secrete anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-35 (Bettini and Vignali, 2009). These regulatory cytokines can affect multiple cell types at the site of inflammation. One mechanism by which Tregs are able to target autoreactive $CD4^+$ T effector memory cells is through the generation of tolerogenic APCs (Figure 1a). When APCs are exposed to TGF-β and IL-10, they express a tolerogenic phenotype that promotes anergy of memory T cells that bind to their MHC molecules (Torres-Aguilar *et al.*, 2010a; 2010b). Tolerogenic APCs are also able to induce IL-10 secreting T cells, but it is unclear whether these T cells were naive or memory cells prior to interacting with the tolerogenic APC. This mechanism allows for the targeting of antigen-specific memory T cells when cells become reactivated by tolerogenic APCs at the site of inflammation.

In addition to causing anergy in memory CD4 T cells and activation of IL-10 secreting Tregs, tolerogenic APCs upregulate programmed death ligand 1 (PD-L1) (Wolfle *et al.*, 2011). The programmed death (PD-1)-PD-L1 signaling pathway is one mechanism essential for the suppression of T cells post activation in autoimmunity (Riella *et al.*, 2012). Post

antigen activation, exhausted $CD8^+$ T cells upregulate the expression of certain cell surface markers, such as PD-1 (Chikuma *et al.*, 2009). This upregulation of PD-1 leaves CD8 T cells susceptible to PD-L1 dependent anergy (Figure 1b). The generation of exhausted $PD\text{-}1^+CD8^+$ T cells involves the depletion or blockade of IL-2 in the environment. Treg express higher amounts of the high affinity IL-2 receptor (CD25) and are capable of depleting local cytokines (Pandiyan *et al.*, 2007). In the case of autoimmunity, Treg cells are expected to soak up IL-2 at the site of inflammation leading to exhaustion of $CD8^+$ T cell and leaving them susceptible to PD-1-PD-L1 mediated cell death.

B cells play a critical role in autoimmune disease pathology via the secretion of autoantibodies. These antibodies bind to endogenous proteins and allow for direct targeting of specific cell types by Fc receptor and complement mediated mechanisms (Yanaba *et al.*, 2008). The effector mechanism of autoantibodies has been verified via adoptive transfer of autoantibodies into animal models in which they exacerbate tissue pathology in an analogous manner as in the human disease (Yan *et al.*, 2014; Saadoun *et al.*, 2010). While regulatory cytokines such as TGF-β induce apoptosis in B cells (Spender *et al.*, 2009), Treg cells are also capable of directly suppressing autoantibody secreting B cells via cell-to-cell contact (Figure 1c) (Lim *et al.*, 2005). Animal model studies have shown that depleting Treg cells leads to increase autoantibody production (Liu *et al.*, 2014). Patients deficient in functional FoxP3, a disease called immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, lack nTreg cells and suffer from a number of serious autoimmune and inflammation disorders that are lethal if not treated by bone marrow transplantation (van der Vliet and Nieuwenhuis, 2007). When Treg cells are activated by B cells that secrete autoantibodies, Tregs can inhibit proliferation of B cells and drive cell death in a perforin and granzyme-dependent manner (Wang and Zheng, 2013).

Imbalance of Regulatory T cells in Human Autoimmune Diseases

While Treg cells are present in patients with autoimmune disorders (Putheti *et al.*, 2004; Viglietta *et al.*, 2004), this population of cells appear to lack auto-antigen specificity as well as suppressive capabilities (Venken *et al.*, 2008; Viglietta *et al.*, 2004; Ehrenstein *et al.*, 2004). While the number of Tregs may vary from patient to patient and differ in activity between various autoimmune diseases, it is clear that the Treg suppressive phenotype is limited within patients with autoimmune disorders (Long and Buckner, 2011). Because of this observation, multiple therapies have been designed and tested to expand Treg cells in animal disease models and in patients suffering with autoimmune disorders. The following section will focus upon notable therapies that increase the number of Treg cells when administered *in vivo*.

IL-2 therapy rescues Treg population

When originally discovered, interleukin-2 (IL-2) was seen as a proinflammatory cytokine, which supported the proliferation and activation of naive T cells into T helper cell subsets (Robb, 1984). Although $Foxp3^+$ T cells are present and functionally suppressive in $IL\text{-}2^{-/-}$ and $IL\text{-}2r\alpha^{-/-}$ mice, IL-2 is an important cytokine in regulating the transcription of growth and metabolic genes in the context of Treg cells (Fontenot *et al.*, 2005). Clinical trials have

begun with autoimmune and inflammatory disorders to examine whether low dose IL-2 treatment promotes the formation of Treg cells in patients (Koreth *et al.*, 2011; Saadoun *et al.*, 2011). Because of their expression of a high affinity IL-2R, it is thought that Tregs may preferentially expand in conditions of low amounts of IL-2. Low dose IL-2 treatment results in an increase in the percentage of peripheral blood $CD4^+CD25^{High}Foxp3^+$ T cells with increased suppressive function. In graft-versus-host-disease (GVHD) patients that received a 12-month treatment with IL-2, researchers observed an increase in the number of $CD4^+$ Treg cells (Koreth *et al.*, 2011). In patients with hepatitis C-induced vasculitis, Treg cell numbers were restored after low dose IL-2 therapy and 8 of 10 patients saw clinical improvement of their autoimmune disorder (Saadoun *et al.*, 2011). It is of note that no effect was observed on T effector cell populations in patients from either of these clinical studies.

Therapies utilizing autoantigens to induce Treg suppression

Screening autoantibodies isolated from the plasma of patients, a large array of autoantigen epitopes have been identified for various autoimmune disorders (Lernmark, 2001). Utilizing this knowledge, methods have been tested to identify procedures that expand antigen-specific Treg cells that are capable of inducing tolerance against autoantigens and inhibiting autoimmune inflammatory events from a polyclonal T cell population (Xu *et al.*, 2013). Autoantigens have been utilized in two different methods to produce tolerogenic T cells *ex vivo*: (i) antigen bound directly to biodegradable particles (Maldonado *et al.*, 2015), or (ii) antigen fixed onto APCs to trigger the formation and expansion of $Foxp3^+$ T cells and the secretion of the regulatory cytokines IL-10 and TGF-β (Prasad *et al.*, 2012b).

Nanoparticle technology has been developed in the past decade to expand T cell populations (Steenblock and Fahmy, 2008; Park *et al.*, 2011). Biodegradable nanoparticles with autoantigens and rapamycin bound to their surface are capable of generating $Foxp3^+$ Treg and B regulatory cells in multiple animal models (Maldonado *et al.*, 2015). Under these conditions, the nanoparticle taken up by APCs led to the presentation of the autoantigens on MHC molecules. The use of rapamycin directed the APCs to express a tolerogenic phenotype that allowed for the activation of inducible Treg cells. Nanoparticle treatment given to mice via subcutaneous or intravenous injection showed promising results in ameliorating disease in the relapsing-remitting multiple sclerosis animal model (Maldonado *et al.*, 2015). This therapy relies on rapamycin bound to the nanoparticle interacting with the APCs. Loss of the rapamycin on the nanoparticle results in the inability to generate the Treg cell population.

Another approach to generate autoantigen-specific Treg cells is by cross-linking autoantigens to the cell surface of APCs with the chemical ethylene carbodimide (ECDI) (Miller *et al.*, 2007). Adoptive transfer of APCs ECDI-crosslinked with diabetes associated autoantigens induces tolerance to antigens and inhibits the development of spontaneous type-1 diabetes (T1D) due to the generation of antigen-specific regulatory T cells in the NOD diabetes model (Prasad *et al.*, 2012a). Additionally, this treatment in concert with islet cell transplantation leads to the restoration of normoglycemia in mice (Kheradmand *et al.*, 2011). Treated mice show an increase in IL-10 secretion when cells were restimulated *ex vivo.* Additional studies show that there is an increase in $CD4^+Foxp3^+$ T cells in the spleen

and draining lymph nodes following treatment (Kheradmand *et al.*, 2012). A first-in-man clinical trial examined the safety of antigen-ECDI-cell treatment in patients with multiple sclerosis (MS) (Lutterotti *et al.*, 2013). MS patients whose T cell responses were specific for myelin peptides were given identical peptides chemically coupled to autologous PBMCs. While the higher doses of the treatment showed a reduction in autoantigen-specific T effector cells, treatment did not appear to have any effect on the peripheral Treg cell population.

Adoptive Treg cell therapy to mediate inflammation

In GHVD and autoimmune animal models, early Treg cell treatment inhibits the development of the disease (Roncarolo and Battaglia, 2007). In Hori *et al.* (2002), researchers utilized a RAG-1 deficient TCR transgenic mouse model with a TCR specific for myelin basic protein (MBP) that spontaneously developed CNS autoimmunity (Hori *et al.*, 2002). Adoptive transfer of MBP-specific Treg cells into this model prior to the development of disease symptoms led to suppression of clinical phenotypes. Additionally, this study also showed that adoptive transfer of non-MBP specific Treg cells was capable of suppressing the disease phenotype. Studies performed in an animal model for type 1 diabetes has shown similar results when autoantigen-specific T cells were adoptively transferred into NOD $Rag^{-/-}$ mice prior to the development of clinical symptoms (Masteller *et al.*, 2005; Tang *et al.*, 2004). These studies suggested that reconstituting the immune system with autoantigen-specific Tregs prior to the onset of clinical disease would prove beneficial at inhibiting the early auto-inflammatory events that initiate the disease. Studies performed in GVHD models suggest that adoptive transfer of Treg cells post reconstitution of the immune system decreases the efficacy of Treg adoptive cell therapy (ACT) (Nguyen *et al.*, 2007). A 2007 study showed that mice who received an infusion of Treg cells on day 0 versus on day 21 post bone marrow transplant had significantly less occurrence of GVHD and a greater chance of survival over the long term (Nguyen *et al.*, 2007). While many of these preclinical studies showed that early adoptive transfer of Treg cells proved most effective in preventing disease, Tang *et al.* (2004) indicated that ACT of Treg cells into the diabetic NOD mouse model during the early stages of diabetes showed efficacy in suppressing the progression of the disease (Masteller *et al.*, 2005). All of these preclinical studies provide some proof-of-concept that ACT of Treg cells could be an effective therapeutic option for autoimmunity and GVHD. The efficacy of the treatment relies primarily on the antigen specificity of the Tregs and whether the cells are given during early or late stages of disease progression, with earlier treatment being more effective.

One of the greatest hurdles to overcome in generating an effective ACT Treg cell therapy in humans is the need to transfer a large number of Treg cells into patients. Treg cells are of a very low percentage of leukocytes in the blood, so isolating enough Treg cells to generate a suppressive response has proven challenging. Several laboratories have generated *in vitro* protocols that expand small populations of Treg cells and maintain their suppressor phenotype (Hoffmann *et al.*, 2004; Putnam *et al.*, 2009). The expanded Treg cells retained their suppressive capabilities (secretion of IL-10 and Foxp3 expression) and had low proinflammatory characteristics (as measured by IFN-γ or IL-4 secretion post activation) (Bluestone *et al.*, 2015). Starting in 2009, multiple early stage clinical trials examined the

safety and efficacy of utilizing ACT Treg cell therapy in GVHD patients and autoimmune disease patients (Bluestone *et al.*, 2015; Brunstein *et al.*, 2011; Desreumaux *et al.*, 2012; Marek-Trzonkowska *et al.*, 2012; Trzonkowski *et al.*, 2009). These first-in-man studies verified that ACT Treg cell therapy is tolerable in humans, with patients showing minimal negative side-effects associated with treatment.

In cases of autoimmunity, treatment with Tregs did not show the same efficacy as what was previously demonstrated in studies using animal models. Bluestone *et al.* (2015) reported a clinical study examining a dose escalation of *ex vivo* expanded polyclonal Tregs and their effect on conditions in early onset type 1 diabetes in adults (Bluestone *et al.*, 2015). Treatment with the highest dose of Tregs (2.6×10^9 cells/patient) showed no significant difference in the progression of disease. This study showed that despite Treg cells having a suppressive capacity post *ex vivo* expansion, adoptively transferred Treg cells did not survive long term in patients. When isolated from the patients, the Treg cells had lost their activation phenotype. The use of antigen specific Treg cells may be critical for efficacy in autoimmune patients. Desremaux *et al.* (2012) reported the use of ovalbumin-specific Treg cells for the treatment of patients suffering from refractory Crohn's disease (Desreumaux *et al.*, 2012). Due to the site of inflammation being predominantly within intestinal tissues, the researchers generated clonal Tregs specific for ovalbumin. They theorized that since ovalbumin is commonly consumed, the antigen would be presented to Treg cells in intestinal tissue. Twenty patients received a single dose of ovalbumin-specific Tregs ranging from 10^6 to 10^9 cells. Of the 20 patients tested, 8 patients showed a reduction of Crohn's disease activity index. Taken together, these two studies suggest that the antigen specificity of the Treg may play an important role in designing future Treg cell based therapies.

ACT of Tregs has been further characterized in studies performed in hematopoietic stem cell transplantation (HSCT), with the goal of preventing GVHD. In patients that receive bone marrow infusions, there is a 30–60% chance that the transferred immune cells will generate an immune response against the host's proteins (Barton-Burke *et al.*, 2008). The immune response of grafted cells against endogenous proteins leads to continuous inflammation and damage to tissues and organs. When treating acute leukemia patients with HSCT, the infusion of allogeneic bone marrow cells is meant to reconstitute the immune system post high dose irradiation treatment (Rezvani and Barrett, 2008). One side effect of HSCT therapy, however, is that donor T cells meant to protect the patients from pathogens will also target host tissues and mediate GVHD despite HLA matching due to the many non-HLA differences between host and donor. Clinical studies have examined whether donor-derived $Foxp3^+$ T cells can prevent GVHD without affecting donor T cell derived protection from infections (Di Ianni *et al.*, 2011; Martelli *et al.*, 2014). Di Ianni *et al.* (2011) showed that 26 out of 28 patients achieved donor-engraftment with only 2 of the 26 patients developing ≥ grade 2 GVHD. Martelli *et al.* (2014) showed that of the 43 patients that received donor Treg cells four days prior to receiving HSCT, 95% donor engraftment was achieved with only 15% of patients developing ≥ grade 2 GVHD. With the positive outcomes being observed-with these studies, other groups have examined additional methods to protect against GVHD following HSCT. In 2014, Bacchetta *et al.* (2014) describes a clinical trial in which patients received HSCT in combination with alloantigen-specific Treg cells. The alloantigen-specific Tregs were generated by culturing donor derived peripheral blood mononuclear cells

(PBMCs) with host APCs in the presence of IL-10. Researchers theorized that ACT of Treg cells would lead to anergy against host-derived alloantigens but still allow for protection against bacterial or viral antigens (Bacchetta *et al.*, 2010). Of the 12 patients given the treatment, 4 patients showed a decrease in alloantigen-specific cytotoxic T cell populations following the adoptive transfer of IL-10 anergized Treg cells. These patients also showed stable donor chimerism and long-term immune reconstitution.

Conclusions

It is possible that one of the major driving forces of autoimmunity is the lack of regulatory T cells against disease-specific autoantigens. Clinical trials in GVHD patients show a positive correlation with the reconstitution of autoantigen-specific regulatory T cells and a decrease in disease progression. The available data do not support the notion that reconstitution of the immune response with polyclonal Tregs can inhibit disease progression. It may turn out that for ACT of Tregs to be of clinical benefit, Tregs must become activated *in vivo* against an antigen present within the auto-inflammatory environment. With the limited number of Tregs found in patients, future studies may examine the use of non-autologous Tregs from haplotype similar individuals or genetically modifying autoantigen-specific T effector cells to become Treg cells themselves. Treg cells that are present and activated during auto-inflammatory disease may be capable of inhibiting the progression of disease.

Disease progression in autoimmune disorders is highly variable between patients diagnosed with the same disorder. For immune suppression to occur in patients suffering from an autoimmune disorder, identifying the opportune stage of disease to intervene may be critical for the efficacy of treatment. For ACT of Tregs to prevent GVHD, Treg cells are transferred along with the bone marrow graft, allowing for the early suppression of T effector cells that can trigger GVHD. Early suppression of inflammatory cells is key for beneficial effects to be observed. For autoimmune diseases, early treatment methods or more effective Treg cells must be generated for ACT of Tregs to become an effective approach to inhibit the progression of clinical disease.

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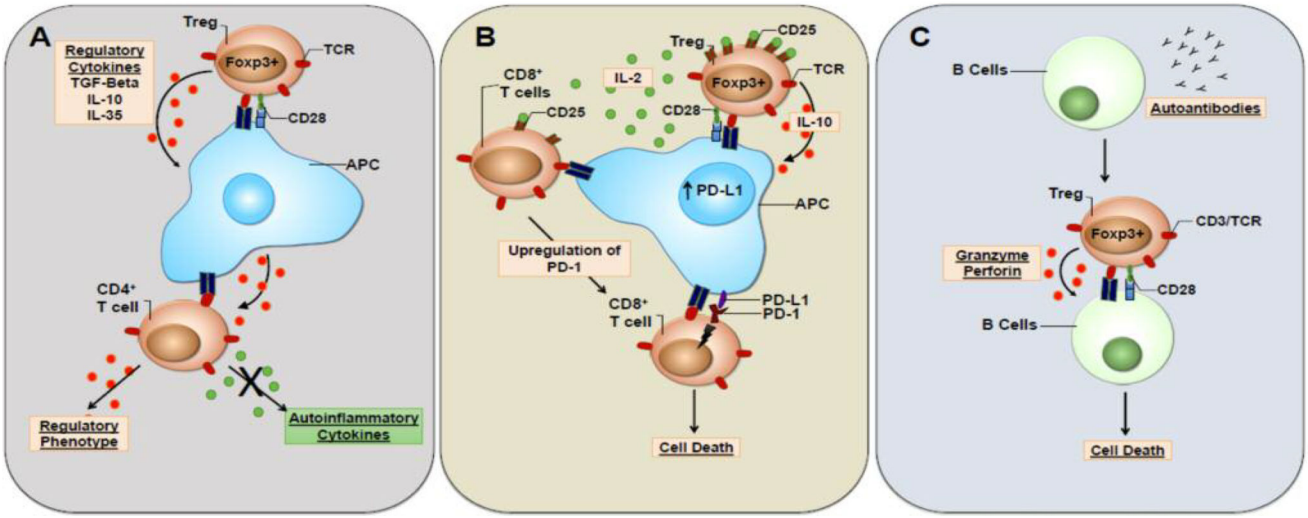

Figure 1.

Treg cell mechanism of suppression in autoimmunity. (A) When activated, Treg cells are capable of driving the formation of tolerogenic APCs via the secretion of regulatory cytokines (e.g., TGF-β and IL-10). Tolerogenic APCs activate antigen-specific CD4+ T cells and skew them towards a regulatory phenotype. (B) IL-2 cytokine depletion drives the upregulation of PD-1 on activated CD8+ T cells. Upregulation of PD-L1 by tolerogenic APCs, leave CD8 T cells vulnerable to cell death in a PD-1/PDL-1 dependent manner. (C) B cells are capable of presenting antigens to Treg cells. Once activated by B cells, Tregs suppress the secretion of autoantibodies via direct killing of autoinflammatory B cells in a perforin and granzyme dependent manner.