

Review

Clinical application of expanded CD4⁺25⁺ cellsCarl H. June^{a,*}, Bruce R. Blazar^b^a The Abramson Family Cancer Research Institute, The Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA^b University of Minnesota Cancer Center and The Department of Pediatrics, Division of Blood and Marrow Transplantation, USA**Abstract**

A central goal immunologists has been to develop targeted therapies that will induce or maintain immunologic tolerance in the absence of potentially harmful immunosuppression. The ability to isolate and expand regulatory T-cell populations with immune suppressive activity will enable new forms of adoptive immunotherapy that may achieve this long held dream. Assuming that certain technical challenges regarding the manufacturing of regulatory T cells can be overcome, a wide variety of clinical applications can be envisioned using adoptively transferred CD4⁺CD25⁺ regulatory T cells. It is likely that suppressor T cells will first be tested for their ability to prevent or treat graft-versus-host disease (GVHD) following allogeneic bone marrow or stem cell transplantation. A related approach will be clinical studies to induce allogeneic or xenogeneic tolerance using regulatory T cells in solid organ transplantation. A more technically challenging approach will be the use of regulatory T-cell therapy for autoimmune disorders. Finally on the horizon are approaches that will use genetically engineered lymphocytes to replace regulatory T cells in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, and potentially to create more potent regulatory T (Treg) cells with enhanced suppressive activity.

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Keywords: Adoptive immunotherapy; Regulatory T cell; Graft versus host disease**1. Introduction**

CD4⁺CD25⁺ T regulatory (Treg) cells have been shown to regulate self-tolerance in mice. Initially described as critical for the control of autoimmunity [1], Treg were found upon adoptive transfer to prevent experimental autoimmune diseases [2]. More recently, Treg have been shown to suppress allogeneic immune responses [3–5] and prevent transplant rejection [6]. Studies of human Treg have been hindered by low numbers present in peripheral blood and were initially thought to have poor replicative capacity *in vitro* [7]. With rare exceptions that can be envisioned,¹ regulatory T-cell therapy will require *ex vivo* expansion of the input T cells, because regulatory T cells are present at low frequency in most sites that are clinically accessible for harvest that could serve as a source for input culture cells. Fortunately, it was recently shown that Tregs have substantial replicative capacity *in vivo* [8–10]. Recently, we and others have developed improved and clinically scalable *ex vivo* culture conditions that should permit pilot trials of Treg-adoptive

immunotherapy for the prevention or therapy of GVHD [11–15]. This now permits in principle, the ability to consider the adoptive transfer of regulatory T cells for diverse human immunotherapies.

2. Principles of adoptive immunotherapy

The idea that passive transfer of primed immune cells can generate immunity in the recipient is a relatively old idea in the history of immunology. First proposed by Billingham et al. [16] in 1954 who coined the term “adoptive immunity”, numerous animal studies have demonstrated the effectiveness of this adoptive transfer of immunity toward cancer and infectious disease (for recent reviews, see [17,18]. The history of adoptive immunotherapy dates back to the first testing of adoptive cell transfer to treat tumors in inbred mice in the 1950s [19]. In humans, the use of IL-2 permitted for the first time the *ex vivo* culture and expansion of T cells [20]. The adoptive transfer of immune cells can have remarkable specificity towards its targets and specificity using strategies such as *in vivo* [21] and *ex vivo* [22,23] priming and genetic engineering [24,25]. Moreover, adoptive immune cell transfer has the potential to induce long-standing effects via the establishment of immunologic memory.

Many lessons can be learned from previous experiments in rodents and humans with adoptive T-cell therapies involving

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¹ For example, monozygotic twins discordant for autoimmune disease.

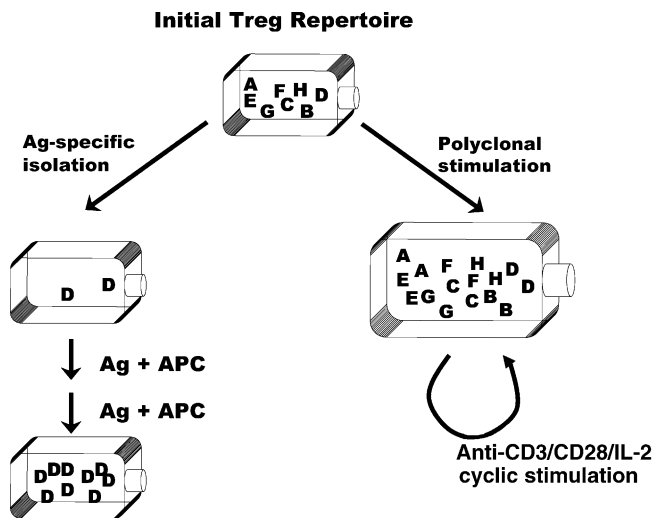


Fig. 1. General approaches for ex vivo Treg cell expansion. Antigen-specific Tregs can be generated from the input repertoire by tetramer selection of Tregs with desired specificity and repeated stimulation with antigen pulsed antigen presenting cells (APC). This process usually requires several rounds of stimulation (left). Alternatively, the starting Treg repertoire can be expanded by polyclonal stimulation via anti-CD3/CD28/IL-2 stimulation to generate polyclonal Tregs (right). At least 27 cell divisions are required from a single precursor T cell in order to generate 1 billion clonal T cells.

the transfer of effector cells for the treatment of cancer and chronic infection. Two basic approaches are being tested for adoptive immunotherapy (Fig. 1). The first approach has been to isolate and activate antigen-specific T cells from peripheral blood or tumor specimens and then clonally expand the antigen specific cells in vitro by various approaches [26–28]. In the second approach, polyclonal ex vivo activation of the input T cells is done [29–31], based on the assumptions that antigen-specific T cells are present in the patient and have been primed in vivo [32,33]. Polyclonal ex vivo activation also may be useful to restore the in vivo dysfunction of the T cells due to the tumor microenvironment [34–40]. The input cells are first activated polyclonally by various means in vitro and are then reinfused with the expectation that they will now respond directly to tumor or to tumor antigen presented by APCs or targets in the patient. The first approach will guarantee antigen specificity but is costly and labor-intensive. The second approach is technically more facile. Both approaches have been strengthened by the realization that homeostatic expansion of transferred lymphocytes can improve engraftment and effector functions in vivo [41]. The rationale for the polyclonal approach has been substantially strengthened by the realization that most patients are already primed to their tumor, and that the major issue is improving the quality and quantity of the natural immune response. Recent clinical trials show the promise of both general approaches and suggest that adoptive therapy in lymphopenic recipients can augment therapeutic effects [42,43]. Potential therapeutic approaches using polyclonally expanded Tregs will be discussed below, and approaches using ex vivo-expanded antigen-specific Tregs are described by Bluestone elsewhere in this volume.

Studies conducted during the past several decades have revealed a number of principles that will be instructive as

immunotherapy with Tregs is contemplated. These principles are to (1) avoid the induction of immunogenicity of the infused cells; (2) prevent or delay cellular immunosenescence; (3) maximize help; and (4) be cognizant of the known differences between mouse and human T-cell biology. It should be self-evident that culture technologies should be adopted that minimize immunogenicity of the infused Tregs [44]. However, it is sometimes unavoidable that the culture process may render cells more antigenic than when in the quiescent state. Given that the immune system is probably the most efficient biosensor system ever developed, it is not surprising that seemingly trivial modifications to cell culture technologies can have large differences in their immunogenicity and subsequent engraftment of the adoptively transferred cells. Immunogenic determinants implicated in poor engraftment include foreign proteins introduced during cell processing and culture, such as fetal calf serum and collagenase [45–47], mouse monoclonal antibodies such as OKT3 [48,49], and self-antigens, such as idiotypes present in very low levels prior to ex vivo expansion. Attention was first brought to the immunogenicity of autologous human cells when lymphocytes were retrovirally transduced with the neomycin phosphotransferase or thymidine kinase together with the hygromycin phosphotransferase gene prior to expansion and reinfusion [27,50]. In retrospect, this seems an obvious obstacle to adoptive cell transfers involving gene therapy. However, the experience in humans at the time was from transfusion medicine, which predicted that autologous cellular products would not be immunogenic. Mouse Treg cells are MHCII-negative and may be less immunogenic, in striking contrast to the bright levels of MHCII that are present on the surface of activated human Tregs. Given that Treg immunotherapy is contemplated in patients with heightened immune systems who various have autoimmune disorders, it will be particularly important to avoid the induction of immunogenicity during the cell manufacturing process. Furthermore, given that multiple infusions of Tregs may be given to patients as maintenance therapy to maintain organ allografts or prevent recurrent autoimmune disease, it will be important that the Tregs be non-immunogenic. The issue of immunogenicity of the cell product is less of an issue for stem cell therapies where only a single infusion of cells is given.

Human T cells have finite clonal lifespans in vitro [51], a phenomenon also manifested with other somatic tissue cells and commonly known as the Hayflick limit [52]. Lymphocytes and stem cells belong to a select class of cells that have the capacity for induction of telomerase activity [53]. It is interesting to note that birds display telomerase repression and telomeric shortening with somatic cell division similar to that seen in humans. However, rodents retain telomerase-competency throughout their lifespan, and their lymphocytes have not been shown to display division-dependent telomere shortening [54]; this presents a major shortcoming in using mice for adoptive immunotherapy studies. Costimulation is required for induction of telomerase activity in effector T cells [55,56]. In vitro, the limit of polyclonal expansion for adult mature CD4 T cells is about 30–40 population doublings [57,58]. In previous studies we found that human naïve CD4 T cells have telomeres on average that are 1.4 kb longer than human memory T cells [59].

The degree of telomere erosion has not been reported in human Tregs; however, it has been demonstrated that ectopic expression of hTERT can extend the proliferative capacity of Tregs in vitro [60]. T-cell receptor excision circles (TREC), an indicator of recent thymic emigrants, are fewer in adult CD4⁺CD25⁺ cells than in the CD4⁺CD25[−] cells in peripheral blood [61], suggesting a more extensive post-thymic replicative history for Tregs on average than for non-Treg cells. It is possible that many previous clinical adoptive immunotherapy trials have been unsuccessful because the prolonged ex vivo culture process resulted in a population of cells that had reached, or were near replicative senescence. Recent studies show that the in vivo persistence and engraftment of tumor infiltrating lymphocytes correlates with telomere length and tumor regression in patients with melanoma [62]. It will be important to optimize culture systems to maintain or enhance telomerase activity for Treg immunotherapy.

Many studies show that the generation and/or maintenance of CD8 memory requires CD4 cell help [63], and clinical adoptive transfer studies show that the persistence of CD8 effector T cells is enhanced with the concomitant administration of IL-2 [64]. Data is more limited with adoptive transfer of human CD4 effector cells. These cells can have prolonged survival that is likely due to the production of autocrine help, and one study in patients with HIV infection has shown that the administration of exogenous IL-2 did not enhance the persistence of CD4 T cells [65]. CD4 effector cells enhance the persistence of CD8 cells in patients with HIV infection and cancer [66,67], particularly when the T cells have been costimulated through anti-CD28 [67]. IL-2 is necessary for the expansion of Tregs in mice following adoptive transfer [68,69]. IL-7 increases Treg expansion in lymphopenic mice, and there appeared to be no preferential advantage for effector CD4 cells under these conditions as there were no differences detected in IL-7-driven expansion of CD4⁺CD25⁺ or CD4⁺CD25[−] cells [70]. Although some currently used culture systems for ex vivo expansion of Tregs use high concentrations of IL-2 [71,72], it is likely that the adoptive transfer of ex vivo-expanded Tregs in lymphopenic humans will provide conditions for sustained engraftment and expansion in vivo most likely without the need for exogenous IL-2. When mice in steady state are given Tregs by adoptive transfer, a subset of the cells rapidly divides, presumably in response to autoantigen, while another subset stably engrafts with minimal division [73]. In humans, it is likely that maintenance therapy with Tregs will be required to sustain therapeutic effects in situations in which there is an ongoing need to suppress detrimental T-cell responses. In contrast to the need for exogenous IL-2 to support Treg expansion in lymphopenic patients, strategies that increase TGF- β signaling may augment the expansion and suppressive functions of Tregs following adoptive transfer [74].

Finally it is important that immunotherapy strategies take into account the differences that have been uncovered between mouse and human Treg. Most aspects of regulatory T-cell biology are highly conserved between the rodent and human immune systems. However, some major differences have been uncovered that are relevant to immunotherapy. These include (1) the induction of a T-regulatory type 1 (Tr1) phenotype by CD46 costimulation in humans but not mice [75]; (2) the existence of

CD8⁺CD28[−] suppressor cells in humans but not mice [76–78]; and (3) the ability of human but not mouse Tr1 cells to have contact-dependent cytotoxicity [79]. CD223 (LAG-3) has been proposed to be a marker and to be required for function in murine Tregs; it is not yet known if CD223 has a role in human Tregs [80].

3. Clinical application of expanded CD4⁺25⁺ cells

3.1. Graft-versus-host disease (GVHD)

GVHD was the first disease that was cured in mice with ex vivo-expanded regulatory T cells [5,81,82]. Addition of freshly isolated Tregs to the donor graft ameliorates GVHD and facilitates alloengraftment [83–85]. The adoptive transfer of Tregs was able to prevent GVHD while preserving graft versus tumor activity [86–88]. For several reasons, GVHD following allogeneic bone marrow transplantation or peripheral blood stem cell transplantation in adults is likely to be the initial setting for the first-in-human trial with ex vivo-expanded and adoptively transferred regulatory T cells. There are several characteristics that make this the most likely starting place for the exploration of the safety and feasibility of adoptive regulatory cell therapy. The first issue is that since regulatory T cells are antigen-specific in that they require TCR signals for maximum expansion and retention of suppressive capacities in vivo, it is desirable that the initial tests be carried out in settings where the repertoire is not limiting and there are abundant target antigens to provide TCR signaling in vivo. This is because the primary technical challenge will be to obtain sufficient numbers of ex vivo-expanded Tregs cells from peripheral blood and in patients with a restricted repertoire, obtaining sufficient numbers of antigen-specific Tregs for expansion and reinfusion will be difficult. The alloreactive repertoire is broad in the setting of allogeneic marrow or stem cell transplantation, and therefore polyclonal Tregs expanded from the peripheral blood should be a sufficient source of alloreactive regulatory T cells. Second in the case of transplantation, the regulatory T cells will be derived from the donor. This has a significant practical advantage in that the cells should exhibit normal replicative potential, whereas the host, as a consequence of an underlying hematologic malignancy as well as having had previous cytotoxic chemotherapy, peripheral blood Tregs will likely have an impaired replicative capacity. It is perhaps relevant that we have noted significant replicative impairments of effector T cells in previous studies of patients with hematologic malignancies who have had substantial pretreatment with cytotoxic chemotherapies [29]. Third, acute GVHD has a predictable time of onset, usually within the first 100 days following transplant, and the incidence and severity of acute GVHD within a given patient population are well known. Thus, it should be possible to determine in a timely fashion if there is efficacy in preventing GVHD, which is a significant cause of morbidity and mortality after transplantation. Fourth, given the extreme lymphopenia of the host following high-dose chemotherapy, it may be possible to determine the engraftment in the peripheral blood of the infused donor Tregs in the host. In previous trials, we have

been able to document an accelerated immune reconstitution that is induced with the adoptive transfer of effector lymphocytes in the immediate post transplant period by counting and phenotyping the lymphocytes [29,43]. It will be useful to document whether the adoptively transferred Tregs engraft and persist, and furthermore, to determine whether a dose response exists. In the steady state settings of other treatment scenarios with Tregs, such as solid organ allografts and autoimmunity, it likely will not be possible to document engraftment of the infused Tregs without resorting to gene marking the cells or perhaps sampling the target organ.

There are, however, several challenges in testing Tregs in marrow allograft recipients. First, the Tregs will be infused into the host who has had tissue damage and inflammation as a result of the pre-transplant conditioning chemotherapy. This inflammatory environment could impair the suppressive function of the Tregs, or their ability to create infectious tolerance in conventional CD4 or CD8 cells [89]. Second, due to practical considerations, the Tregs will be tested in the presence of pharmacologic levels of immunosuppressants, since it is not ethical to carry out allogeneic transplantation in which high numbers of donor T cells, contained in the donor graft, are given without also providing prophylactic immunosuppression [90]. The choice of immunosuppressant is critical, as various drugs in clinical use can potentially antagonize or synergize with the Tregs [91,92].

3.2. Autoimmunity

Regulatory T cells first were discovered based on their ability to prevent a number of autoimmune disorders in mice [93]. A congenital deficiency of FoxP3 in humans leads to a deficiency of Tregs and is associated with a variety of autoimmune immunopathologies [94]. Thus, it is likely that Tregs will have a therapeutic role in some forms of human autoimmunity. It will be important to select the optimal patient subsets for therapy, both in order to avoid toxicity and to enhance the likelihood of therapeutic success. By reasoning from analogy in cancer, where the tumors that are characterized with spontaneous remissions such as renal cell carcinoma and follicular lymphoma tend to be highly responsive to immunotherapy, it may be best to consider first treating human autoimmune diseases that are characterized by spontaneous remissions and exacerbations. Thus, it may be fruitful to investigate the effects of adoptively transferred regulatory T cells in the relapsing form of multiple sclerosis, and systemic lupus erythematosus and in an inflammatory bowel disease such as ulcerative colitis and Crohn's disease.

Substantial preclinical evidence has accumulated to indicate that ex vivo-expanded regulatory T cells have the potential to treat SLE [95], multiple sclerosis [96], inflammatory bowel disease [97] and to prevent type 1 diabetes [71,98,99]. However, some challenges will have to be overcome in order to maximize the likelihood of clinical translation. In the case of SLE, the pathogenic antigens remain incompletely defined, so that it is likely that polyclonal populations of Tregs will have to be used [95]. There is some evidence that nucleosomal-specific Tregs can have therapeutic benefit in murine lupus [100]; however,

the applicability of this to the more complex human syndrome remains unknown. Furthermore, it has yet to be determined to what extent deficiencies in numbers or function of regulatory T cells might contribute to the pathogenesis of lupus. There is a stronger rationale for Treg therapy of disorders with qualitative or quantitative impairments of Tregs. At present, this includes patients with multiple sclerosis, rheumatoid arthritis, type 1 diabetes and autoimmune polyglandular syndrome type II [101–104]. Tr1-like regulatory T cells may be less frequent in patients with organ-specific autoimmune illness [105]. If impaired Treg function does not contribute to the immunopathogenesis of the autoimmune syndrome, then the therapy will have to rely on the ability of the adoptive transfer strategy to achieve pharmacologic increases in the number of Treg for a sustained therapeutic benefit. The lymphopenia that is characteristic of lupus and some other autoimmune disorders is likely to induce homeostatic proliferation of Tregs following adoptive transfer. Whether this will favor induction and maintenance of tolerance remains unknown, as there is evidence to support potentially beneficial or detrimental effects [69,106,107]. Considerations related to the immunotherapy of type 1 diabetes are addressed in detail elsewhere in this issue by Bluestone.

3.3. Organ transplantation

Solid organ transplantation such as kidney, pancreas, liver, lung and heart allograft procedures could all potentially benefit from a non-toxic mechanism to induce donor-specific tolerance [6]. Overall survival rate for transplanted organs remains less than 50% at 10 years [108], and thus the induction of immune tolerance that alleviates the need for systemic immunosuppression and decreases the occurrence of late allograft failure represents a major goal of transplantation immunology. Organ transplantation shares with GVHD prevention strategies the opportunity to use donor Tregs to induce tolerance, and a defined onset of immune challenge at the time of surgery. These settings are unlike the situation in autoimmunity where the patient has to serve as the donor of the Tregs, and the patient will already have sustained loss of tolerance due to autoimmunity that results from generally unknown defects in the cellular immune system. With respect to organ grafting, a deficit of Tregs leads to accelerated rejection of skin grafts in mice [3]. Regulatory T cells have been shown to be capable of preventing organ allograft rejection while preserving antiviral immunity in immunocompetent hosts [109]. Both CD4⁺25⁺ and CD4⁺25[−] regulatory cells appear to be able to mediate organ allograft tolerance [110]. Tregs accumulate at the site of tolerated allografts in mice and non-human primates [111,112]. It is anticipated that Tregs eventually will be tested in the setting of both living-related and cadaveric organ donation, assuming that safety and feasibility is demonstrated in the setting of marrow transplantation and GVHD.

The immune response that mediates rejection of organ allografts is unique in that direct antigen presentation of the alloantigen is a dominant mode of triggering alloreactive T cells in the early post graft period, while later, indirect presentation of alloantigen on host APCs is important as well [113]. The balance of direct and indirect alloantigen presentation varies with

the procedure, with liver and skin allografts typically rich in passenger leukocytes that can directly activate host T cells, while pancreatic islet grafts are relatively devoid of these cells. This has important implications on the approach for generating Tregs *ex vivo*; in the case of direct antigen presentation, an approach using host APCs to activate and expand alloantigen-specific donor Tregs would be preferred, while in indirect presentation, the donor Tregs would be activated by donor APCs that have been loaded by various means with host alloantigens and as such a different *ex vivo* culture strategy would be used.

3.4. Infertility

Multiple mechanisms operate to ensure fetal allograft tolerance, including the indolamine 2,3 dioxygenase system [114]. There appears to be a contribution from Tregs in maternal tolerance to the fetus [115], and some cases of chronic spontaneous abortion are due to sensitization with paternal alloantigens. Pre-clinical studies with paternal Tregs that are specific for maternal alloantigens can prevent abortion in mice [116], and presumably, this approach might be useful to promote full-term pregnancies when used as an immunotherapy for women with recurrent spontaneous abortion.

4. Technical considerations for Treg therapy

4.1. Anatomic source of Treg cells

A number of procedures may be used to obtain precursor cells for Treg-adoptive cell therapy. Initial trials will start with peripheral blood CD4 T cells for input cell culture [11,13]. Umbilical cord blood CD4 cells work well to generate alloantigen-specific Tregs [117–119]. However, based on the principle that antigen-specific T cells tend to accumulate at sites of inflammation, it may be that for example, CD4 cells obtained from synovium or pancreatic draining lymph nodes could be useful for rheumatoid arthritis or type 1 diabetes. The thoracic duct is a rich source of T cells and has been used as a source for the adoptive transfer of effector T cells [120], and whether it contains Tregs or precursor cells for Tregs in humans has not been explored. Studies in the rat suggest that the thoracic duct might be a useful source of Tregs [121].

4.2. Culture systems

Regulatory T cells were initially thought to be differentiated to a post mitotic state. Recent studies have shown that as with effector T cells, Tregs can be induced to proliferate with APCs. However, Tregs have a lag phase of 3–4 days in culture before entering S phase, while effector T cells enter S phase within 1 day of stimulation. The prolonged lag phase probably accounts for the initial notion that Tregs were post mitotic, and this is associated with altered signal transduction pathways [119]. Natural and artificial APC have been effective at inducing sufficient levels of proliferation to support clinical scale adoptive transfer experiments. Both mature and immature DC have been reported to stimulate *ex vivo* Treg expansion [12,98,122–124]. Repeti-

tive stimulation of CD4 T cells with immature human dendritic cells induces suppressor Treg [122,125]. Despite their obvious potential, *ex vivo* approaches using natural DCs to expand T cells for adoptive immunotherapy have been hampered by difficulties in obtaining large numbers of these terminally differentiated, short-lived cells in a GMP-compliant manner. Artificial APC coated with anti-CD3 and anti-CD28 mabs and IL-2 are efficient for *ex vivo* expansion of mouse and human polyclonal Treg [11,13,71,72]. HLA class II-specific tetramers have been used to isolate Tregs specific for influenza hemagglutinin after *in vitro* expansion [126]. Antigen-specific Treg from NOD mice were efficiently expanded *in vitro* using beads coated with peptide-loaded MHC class II mimics and anti-CD28 antibody [127]. The Tr1 subset of Tregs can be efficiently expanded from human but not mouse naïve CD4 cells after stimulation with anti-CD3 and anti-CD46 (membrane complement protein) in the presence of IL-2 [75].

Several TNF family members are prominently expressed on Tregs, including GITR and OX40 (CD134). There may be a role for OX40 in the development and homeostasis of Treg cells because there are reduced numbers of the cells in the spleens of OX40-deficient mice, and there are elevated numbers of Tregs in the spleens of mice that overexpress the OX40 ligand [128]. There is evidence that OX40 ligation can enhance Treg proliferation [128]. Other data suggest that triggering OX40 (CD134) or 4-1BB (CD137) blocks the inhibitory activity of mouse Treg cells [129,130]. Finally, several reports suggest a role for Notch signalling in tolerance through increased generation of Tregs [117,131]. Epstein–Barr virus-transformed lymphoblastoid cell lines transduced with Jagged1 inhibit proliferation and cytotoxicity of EBV-reactive effector T cells [132,133].

In vivo observations that immature DC tend to promote tolerance and mature DC generate effector responses are relevant to the biology of Treg expansion *ex vivo* [134]. In cell culture, the strength and duration of signal can have effects on the relative growth of effector and regulatory T cells. For example, when keeping the agonistic antibody constant, presentation to T cells in soluble or immobilized format can have important effects on the outcome of cell cultures [135]. In addition, the precise antibody binding site and avidity of antibody binding to receptors is important, as some anti-CD3 or anti-CD28 antibodies are more efficient at generating or propagating Tregs [136,137].

The use of a number of soluble agents can promote the selective growth of Tregs in some circumstances. Steroid hormones such as 1,25-dihydroxyvitamin D3 have long been recognized to be immunosuppressive [138,139], and administration of Vitamin D3 analogues can promote Tregs *in vivo* [140]. Estradiol increases FoxP3 expression *in vitro*, and treatment of mice with estradiol increases the numbers of Tregs *in vivo* [141]. TGF- β has been shown to cause modest expansion *in vitro* of Treg from CD25[−] precursor cells [142,143]. However, data regarding the role of TGF- β for the *in vivo* function of Treg are controversial [144–148]. In mice, TGF- β induces a regulatory phenotype in CD4⁺CD25[−] T cells through the induction of Foxp3, which downregulates the inhibitory Smad7 protein that is normally induced by TGF- β and limits TGF- β signaling

[149]. Among currently used immunosuppressants, rapamycin and not cyclosporine has promise to promote selective expansion of Tregs [91,92].

Signaling pathways are altered in Tregs compared to their effector counterparts. The level of CD3 expression is lower in human Tregs [150]. Human Tregs derived from cord blood have normal TCR-mediated activation of Rap1 and Akt, while activation of Ras, mitogen-activated protein kinase 1/2, and extracellular signal-regulated kinase 1/2 is impaired [119]. IL-2 signaling is required to maintain Treg fitness [151,152]. Mice deficient in NF kappaB-inducing kinase (NIK) have Tregs with a hyperresponsive phenotype [153]. Tregs do not appear to be dependent on the protein kinase Ctheta pathway of NF-kappaB activation for TCR-induced responses [154].

Treg cells are not a homogenous cell population and subsets of cells contained in the CD4⁺25⁺ fraction comprise those cells with the most potent suppressive capacity. As cell surface markers are defined that identify the most potent subsets of Tregs, it will be important to adapt culture conditions to best preserve those subsets. For example, cyclosporine has been shown to adversely affect the preservation of the CD27 subset of human Tregs that are more potent suppressor cells than the CD27 negative subset [92]. Tregs must home in vivo to the proper sites to control adverse responses. For suppressing GVHD, secondary lymphoid organ homing of Tregs early after transplant is critical, therefore, Tregs that express high levels of L-selectin (CD62L) and other homing receptors that direct Tregs to those organs will be more effective than Tregs that express low levels of these receptors [85,155]. Thus, expansion protocols that preserve the Treg expression of CD62L and CD27 may be more effective than those that do not [118]. However, it is important to note that the particular homing and chemokine receptor expression profile that is needed will depend upon the disease being treated with Tregs and therefore, the expansion protocols may need to be altered for this purpose.

5. Genetically modified Tregs

Tolerance induction using ex vivo approaches will likely incorporate gene-modified Treg strategies. Genetic modification of T cells can be used to overcome intrinsic limitations by incorporation of novel receptors, resistance mechanisms, and control genes. An approach to circumvent the limited numbers of natural Tregs with the appropriate TCR specificity is to engineer retargeted Tregs. One laboratory has treated experimental allergic encephalomyelitis with gene-modified T cells that express a chimeric receptor that links MHC extracellular and transmembrane domains with the cytoplasmic signaling tail of TCR- ζ [156]. Another laboratory created nucleosome-specific regulatory T cells for therapy of murine lupus by triple gene transfer of TCR- α , TCR- β , and CTLA4Ig [100].

Boys with the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome have genetic disruption of Foxp3 [94]. It is likely that allogeneic marrow transplantation will cure this severe disorder, and it is possible that gene correction of autologous hematopoietic stem cells could be curative, similar to severe combined immunodeficiency that results from

mutations in the common gamma chain or adenosine deaminase [157,158].

Based on findings that FoxP3 appears to be a transcription factor that serves to direct T-cell development to Tregs [159], it may be possible to engineer Tregs by reprogramming conventional T cells. Mouse T cells have been gene-modified to achieve ectopic expression of FoxP3 [159,160–163], and this recapitulates many features of Tregs. Two studies report that human T cells can be reprogrammed by ectopic expression of FoxP3 to display suppressive Treg functions [164,165]; however, induction of FoxP3 expression alone is not sufficient to induce full Treg suppressive activity in human T cells [166]. It is likely that this strategy may be more effective with less committed T cells such as thymocytes and naïve T cells rather than with highly specialized effector T cells.

6. Issues

The principal issue with most applications of adoptive therapy is limitations in cell numbers. Unfortunately, only small numbers of Tregs circulate in human peripheral blood. In humans these cells consist of the fraction of cells that stain brightly for CD25⁺, and account for 1–2% of CD4⁺ cells [167]. Using antigen-specific Tregs, only 5000 ex vivo-expanded Tregs were required to prevent diabetes in the NOD model [98], and when polyclonal ex vivo-expanded Tregs were used, about $1\text{--}2 \times 10^6$ Tregs (1:1 ratio of Tregs with conventional T cells) were required to prevent GVHD [5]. Applying these preclinical results by scaling to body weight would predict that on the order of 1×10^7 antigen-specific Tregs and $<5 \times 10^9$ polyclonal ex vivo-expanded Tregs would be required for immunotherapy. With effector CTLs, typically 1×10^8 to 1×10^9 are sufficient to protect immunodeficient patients from chronic viral infections [26,168]. For practical considerations, in a typical apheresis, approximately 5×10^9 peripheral blood T cells are obtained, and if the Tregs are isolated with ~50% efficiency, then 2.5×10^7 input bright CD4⁺CD25⁺ cells would be expected. Given the current 100- to 1000-fold expansion that has been reported in tissue culture experiments [11,13], it should be feasible to obtain sufficient Tregs for human adoptive Treg therapy.

Another principal concern is the potential adverse effects associated with adoptive Treg therapy. To date, adoptive transfer of effector CD4 cells in patients with cancer and HIV has been safe [18,30]. The primary concern is whether Tregs will induce systemic immunosuppression, based on the fact that while activation of Tregs is antigen-specific, once activated, they can display antigen non-specific suppression [11,169]. It is perhaps instructive to consider patients with advanced malignancy, which is the best-documented instance of acquired gain of function in Tregs in humans. In patients with various tumors, upwards of 30% of the tumor infiltrating lymphocytes have phenotypic and functional characteristics of Tregs, and many cancer patients have increased numbers of Tregs circulating in the blood [170,171]. Approximately 50% of patients with advanced cancer are immune suppressed as assessed by cutaneous anergy, loss of memory responses, and the inability to prime to neoantigens [172–174]. Many of these features may be attributed to tumor-

induced excessive Treg function. It is reassuring that despite the vigorous Tregs in cancer patients, they usually do not acquire opportunistic infections without other defects in the immune system such as lymphopenia and granulocytopenia induced by cytotoxic chemotherapy.

7. Summary

As the field of immunology has exploded, the potential for therapeutic manipulation of immunity through ex vivo culture and genetic manipulation of regulatory T cells holds great promise. Expansion of T cells forms a core process in most T-cell adoptive immunotherapy protocols. This is a central issue with regulatory T-cell therapy that must be addressed by the field to realize the therapeutic promise. We must not lose sight of the reality that seemingly trivial changes in cell culture technologies have major effects on T-cell engraftment, proliferation, trafficking and function.

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