FOCUS: IMMUNOLOGY AND IMMUNOTHERAPEUTICS

Learning to Live Together: Harnessing Regulatory T cells to Induce Organ Transplant Tolerance

Andrew Y. Chang* and Nupur Bhattacharya

Stanford University School of Medicine, Stanford, California

The discovery of immune cells with regulatory effects has created considerable excitement for their potential use in inducing tolerance to transplanted tissues. Despite the fact that these cells possess essential functions *in vivo*, attempts to translate them into effective clinical therapies has proved challenging due to a number of unanticipated complexities in their behavior. This article provides a broad summary of research done to understand the largest of the regulatory cell subtypes, namely CD4+Foxp3+ Regulatory T cells (T_{Regs}†). Special attention will be paid to current and future difficulties in using T_{Regs} clinically, as well as room for improvement and innovation in this field.

INTRODUCTION

Though the concept that cells with the ability to downregulate the immune response has been around for a considerable time, their existence was strongly debated until the early 1990s, when a series of studies by Sakaguchi et al. described a population of CD4+ T cells expressing the IL-2 receptor CD25. When athymic mice were inoculated with T cell transfers depleted of these CD25+ cells, they developed severe autoimmunity in multiple organ systems [1]. Replacement of CD4+CD25+ cells suppressed the disease [1]. These "regulatory" T cells were subsequently suggested to be decreased in human patients with autoimmune diseases such as multiple sclerosis [2].

*To whom all correspondence should be addressed: Andrew Y. Chang, Stanford University School of Medicine, Stanford, CA; E-mail: aychang@stanford.edu.

†Abbreviations: BMT, Bone Marrow Transplantation; CD, Cluster of Differentiation; CTLA, Cytotoxic T-Lymphocyte Antigen; Foxp3, Forkhead Box P3; HLA, Human Leukocyte Antigen; IDO, Indolamine 2,3-dioxygenase; IL, Interleukin; IPEX, Immunodysregulation, Polyendocrinopathy, and Enteropathy, X-linked; LIF, Leukemia Inhibitory Factor; NK, Natural Killer; TCR, T cell Receptor; TGF, Transforming Growth Factor; T_{Reg}, Regulatory T cell.

Researchers immediately recognized the therapeutic potential of these cells, not only in autoimmune disease, where their numbers were presumably decreased (and tolerance thus broken), but also in certain infections and tumors. The peripheral blood of epithelial cancer patients has elevated circulating regulatory T cells, and numerous mouse models have shown that manipulation of this cell population can increase or decrease immune-mediated tumor rejection [3,4]. Their tolerogenic effect also has been hypothesized to underlie the persistence of certain viral infections such as hepatitis C [5].

Particular interest in their ability to determine patient tolerance to non-self antigens was augmented by the discovery that antigen-specific CD4+ regulatory T cells were increased in mice, which tolerated allografted tissues long-term [6]. A number of human studies have since shown that a high number of circulating T_{Regs} in kidney and liver transplant patients is correlated with the stability of graft acceptance [7,8,9]. As such, considerable excitement about the clinical usage of T_{Regs} in organ transplantation has been drawn up in the past decade.

Nevertheless, a number of difficulties have arisen concerning the translation of these observational studies into useful human therapies; the system is much more complex than was initially expected. For example, the heterogeneity, plasticity, and context-dependent activity of T_{Regs} have all stood in the way of developing an effective, yet safe, treatment option for transplant patients. In this review, we summarize the biology of CD4+ Foxp3+ T_{Regs} and then discuss a framework for creating appropriate therapies in relation to the challenges presented. New approaches to apply these concepts in medicine also will be highlighted.

CURRENT LANDSCAPE ON COMBATING TRANSPLANT REJECTION

The transplantation of donor tissues has been the dramatic last resort for intractable end-organ failure in a host of human diseases. Since the 1920s, however, physicians have observed rejection of foreign grafts, no doubt mediated by the immune system's recognition of non-self protein targets [10]. Though a full discussion of the myriad mechanisms by which this process occurs is beyond the scope of this review, the major pathways are due to effector lymphocyte priming against donor HLA antigens, leading to cytotoxic effects (both direct cell-cell or humoral) on the parenchyma or vasculature of the graft [10]. The end result is progressive organ failure.

Before the discovery of regulatory immune cells and their role in promoting tolerance, the goal of creating durable organ transplant survival was focused on the elimination of the effector cells hostile to transplanted antigens. This concept is exemplified in the current repertoire of anti-rejection pharmaceuticals in clinical use, such as cyclosporine, a calcineurin inhibitor. Unfortunately, these therapies have a number of flaws preventing them from becoming acceptable permanent solutions to safe, long-term organ transplant acceptance. First, the drugs are nonspecific, generally dampening the immune system and leading to dangerous immunosuppressive side effects. Second, they are only a short-term solution; patients rarely achieve permanent tolerance and are dependent on these drugs ─ and their side effects ─ for life.

The recognition of T_{Regs} has not only significantly altered the extant paradigm, but proposed an additional reason why long-term tolerance cannot be achieved with our current treatment modalities: the fact that these drugs do not distinguish between effector and regulatory players means that though the anti-graft response is prevented, so is the pro-graft tolerance response [11]. The ideal transplant induction therapy is thus one that Spoerl and Li define in their 2011 review as stable, self-perpetuating, and donor antigen-specific — factors that do not describe our treatments at the moment [12]. Taken together, these observations strongly suggest that more research must be conducted in order to understand how to

heighten the activity of T_{Reg}s in transplant patients, either adoptively or endogenously.

REGULATORY T CELLS

Much work has been carried out since the first studies examining broad CD4+ populations to characterize cells with regulatory properties. One of the first observations has been that there are numerous groups and subgroups of cells (both found *in vivo* and experimentally induced) with suppressive phenotypes of various potency including CD4+CD25+Foxp3+, CD8+Foxp3+, Tr1 cells, Tr35 cells, CD3+CD4-CD8- "Double-Negative" cells, and NKT cells [13,14,15]. For the purposes of this review, the term T_{Reg} will be defined as CD4+Foxp3+ cells, since they are the most numerous, naturally occurring, and well-studied of these various cell types.

The emphasis on CD4+Foxp3+ cells was heightened by the discovery of the Foxp3 (forkhead box P3) transcription factor, which regulates the initiation and maintenance of suppressive properties in T_{Reg}s. The importance of Foxp3 was demonstrated dramatically in *scurfy* mice, which are Foxp3-deficient. These animals have a lack of functional T_{Reg}s and suffer severe autoimmune effects in multiple organs [16]. A related condition in humans known as IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked) is also associated with a mutated Foxp3 gene [17].

CD4+Foxp3+ T_{Reg}s are divided into two subgroups: "natural" TRegs (nT_{Reg}) and "induced" TRegs (iT_{Reg}). nT_{Reg}s are born in the thymus and are selected there by their specificities to self autoantigens, analogous to the process used to select effector T cells in the same organ [18]. iT_{Reg}s, on the other hand, come from existing CD4+Foxp3- T cells in the periphery that have been converted to tolerate, rather than to reject, their target antigen [19]. Their surprising origin implies not only that foreign antigens can become tolerated, but tolerance itself is a fluid, non-static process that is heavily context-dependent. In fact, these non-regulatory T cells are converted to iT_{Reg}s by a number of different factors, including TGF-β, IL-2, retinoic acid and leukemia inhibitory factor (LIF) [20,21,22]. Furthermore, the fact that both types of T_{Reg}s undergo a specificity-mediated selection process means that they are highly specific for individual antigens through engagements with their T cell Receptors (TCRs).

As for how T_{Reg}s suppress their target cells, the mechanisms are likely multiple and, as yet, not fully understood. For instance, it is known that within *in vitro* model systems, T_{Reg}s influence a whole host of immune subtypes, including CD4+ T cells, CD8+ T cells, natural killer T (NKT) cells, and B cells [23]. Their effect is not limited simply to effector cells, however, antigen-presenting cells such as dendritic cells and macrophages are also under T_{Reg} purview, as are osteoblasts, mast cells, and natural killer (NK) cells [23]. Their molecular toolbox for achieving their actions is thus similarly diverse, employing secreted suppressor cytokines (e.g., IL-10, TGF-ß, IL-35), consumption of local activating cytokines (e.g., IL-2), cell-surface molecule signaling (e.g., Galectin-1), and direct cell-cell killing (via the granzyme complex) [23]. Recent findings also show that T_{Reg}s are capable of altering cell surfaces by trans-endocytosing CD86 and CD80 co-stimulatory ligands on target antigen-presenting cells [24]. This is achieved by T_{Reg} CTLA-4, which recognizes those molecules and causes them to be internalized and digested by the T_{Reg}. Another feature of particular interest to transplant physicians in T_{Reg}s is that they also exhibit anti-inflammatory and anti-tissue remodeling effects, including the inhibition of transplant vasculopathy, a condition that accelerates the rejection of a donor organ [25].

CURRENT USE OF T_{Reg} THERAPY IN TRANSPLANT MODELS

The potent aforementioned properties of T_{Reg}s have attracted researchers to begin animal and preliminary clinical tests to bring a therapy closer to reality. A number of murine studies have shown that T_{Reg}s can be

generated in different ways and confer allograft tolerance [25,26,27,28]. Regulatory T cells induced *in vivo*, *in vitro*, or expanded *ex vivo* produced some impressive results, including beating heart graft survivals past a 100-day observation period [25]. Most of these studies, however, have had several key limitations. First, the animal subjects had their existing immune systems downregulated in some fashion, either by sublethal irradiation or the depletion of lymphocytes or CD4 T cells with monoclonal antibody pretreatment. Second, although most of these studies were able to stave off acute transplant rejection, chronic rejection still occurred, or in the absence of frank chronic rejection, histological evidence of inflammatory infiltration in the graft was still noted.

Nevertheless, the possibility of using immunoablation followed by bone marrow transplantation (BMT) as a means of "resetting" the immune system and transferring tolerance to solid organ transplants has led to several small human trials with promising results [29,30,31]. Co-transplantation of both bone marrow and kidneys into patients showed not only cases of long-term graft acceptance, but the complete discontinuation of immunosuppressive therapies for some. As for the association between BMT and T_{Regs}, the Kawai et al. study also detected that Foxp3 mRNA levels in renal biopsies of stable immunosuppression-free patients were about 6 times higher than those from the stable-with-immunosuppression group [31]. Thus, even in the absence of more specific immune-tailoring, fostering the growth of endogenous T_{Regs} or possibly transferring *ex vivo* T_{Regs} could be beneficial to human patients as well.

METHODS FOR HARVESTING AND EXPANDING T_{Regs}

Creating a feasible, more specific T_{Reg} therapy for human transplant tolerance is primarily limited, however, by the difficulty of expanding T_{Reg} populations to sizes large enough to tip the effector-regulatory balance. They are not particularly numerous;

CD4+25+ regulatory cells constitute only 5 to 10 percent of peripheral CD4$^+$ T cells [32]. To this end, a number of experimental strategies are being investigated for ways to grow T_{Regs}, both *in vitro* and *in vivo*. The first major approach involves identifying samples highly purified for naturally occurring T_{Regs}. Since Foxp3 is an intracellular molecule, it is not available as a cell surface marker. As such, a cocktail of antibodies (to CD25, CD45RA, CD27, CD39, CD49b, FR4, or PD-1), with magnetic microbeads and columns, must be used to select them [33]. Thereafter, they can be expanded *ex vivo* using donor or recipient antigen-presenting cells (APCs) or anti-CD3/CD28 coated beads [33]. The resultant T_{Regs} can be reintroduced into the patient. The second method involves the conversion of isolated effector CD4+ T cells into induced T_{Regs}. This can be achieved by exposing them to a whole host of cytokines, costimulatory factors, and growth factors, including TGF-ß, IL-2, LIF, PD-1/PD-L1, retinoic acid, and IDO [20,21,22,34,35,36]. As with the prior strategy, these converted *ex vivo* T_{Regs} can then be administered to the patient.

The third major approach would be to expand T_{Regs} *in vivo* with a variety of growth signals, a process that is made more attractive because it avoids the difficulties of purifying T_{Regs} and then coaxing them to expand *in vitro*. However, though this method seems as simple as introducing growth factors to the patient, the signals are not specific for just T_{Regs} and may result in systemic side effects. A possible workaround is to somehow preferentially encourage the growth of regulatory T cells over effector T cells; indeed, inhibiting effector T cell costimulatory pathways such as CD28, CD40, OX40, ICOS, and CD27 with monoclonal antibodies has been able to induce tolerance to solid organ transplants in murine experimental systems [37].

ADDITIONAL CHALLENGES AND OPPORTUNITIES

In addition to the issue of efficiently generating a large number of T_{Regs} for ther-

apy, several factors inherent to their physiology make it challenging to envision regulatory T cells in clinic in the near future. For one, the difference between natural n T_{Regs} and induced iT_{Regs} described above could have significant ramifications in approaches that exclusively use one subset of T_{Regs} or the other. nT_{Regs}, because they are tailored to recognize and accept self antigens, may cause immunodeficiencies if expanded beyond their normal repertoire of tolerance. iT_{Regs} avoid this problem but suffer from another unique property: They are far more unstable than their nT_{Regs} brethren. iT_{Regs} are much more likely, under various stresses, to lose Foxp3 expression, a process probably due to epigenetic differences [38]. In fact, iT_{Regs} have been noted to turn into anti-tolerant TH17 T cells when exposed to inflammatory cytokines, the direct opposite of properties desired in transplant therapy [39].

Furthermore, one must not forget that T_{Reg} activity is the suppression of an existing active process; much of their effectiveness is due to the fact that they can systematically shut down effector cells. As such, effector cells can find ways to evade T_{Regs}-mediated suppression. One example is memory T cells, which maintain a certain resistance to the activities of regulatory T cells [40]. NK cells, on the other hand, take a more direct approach by lysing T_{Regs} [41]. As such, therapies that seek to use T_{Regs} for tolerizing patients to transplanted organs also will need to overcome the anti-suppressive impulses of other immune cells. For example, Afzali et al. suggest that the resistance of memory T cells to downregulation can be counteracted by infusing T_{Regs} prior to transplantation, thus preempting the development of these resilient cells [40]. NK cells could (cautiously) be targeted for depletion with monoclonal antibodies.

Even if T_{Regs} are able to be easily expanded and the anti-regulatory response sufficiently reduced, more theoretical challenges still exist. First, there is the risk of uncontrolled adoptive T_{Reg} proliferation; inadvertent suppression of the normal immune response may cause unregulated growth of infectious agents and tumor cells.

A possible way around this problem is the engineering of self-limiting or self-destructing T_{Regs} that stop growing after the therapeutic goal is achieved. It is yet an unexplored field, but a cell-surface receptor sensitive to the tolerance-immunocompromise balance (perhaps via circulating cytokine detection) could be coupled to the apoptotic pathway of a T_{Reg} to maintain an appropriate population size. Another unresolved issue is that of crosstalk between the numerous regulatory T cell subsets named above (CD8+Foxp3+, Tr1 cells, Tr35 cells, CD3+CD4-CD8- "Double-Negative" cells, NKT cells). Immunosuppression is a sophisticated tightrope to walk, and it is highly unlikely that the different suppressor cell types do not communicate with each other to decide this important concern. As of yet, there is little experimental investigation into this subject.

Lastly, there is the issue of T_{Regs} and the current practice of medicine. As was discussed before, the extant standards of treatment involve the use of broad immunosuppressive pharmaceuticals. Calcineurin inhibitors like cyclosporine suppress TCR signaling, blocking the conversion of effector T cells into induced T_{Regs} [42]. All is not lost, however, as other drug classes such as mTOR inhibitors (exemplified by rapamycin/sirolimus) have shown surprisingly positive effects on the development of tolerance [42]. A mouse model utilizing rapamycin, costimulatory blockade, and BMT showed that the therapy could induce mixed chimerism and subsequent graft tolerance without the need for dangerous immunoablative therapies [43]. On the human side, renal transplant patients receiving low-dose rapamycin had increased circulating T_{Regs}, suggesting that future immunosuppressive drug regimens should take into account those that are more T_{Reg}-friendly [44].

CONCLUSIONS AND OUTLOOK

It is undeniable that regulatory T cells are a powerful and important cellular actor in the establishment of tolerance in the

human body. Nevertheless, major strides need to be made in overcoming both technical and mechanistic challenges to turn the existing research into a coherent and specific therapy for transplant patients. Particular attention needs to be paid to expanding T_{Regs} efficiently, controlling their fickle suppressive-inflammatory duality, and overcoming endogenous resistance to regulatory action. If these goals are achieved, a novel and considerable force will have been recruited not only for the treatment of transplant patients, but the understanding and future conquest of autoimmune diseases, cancer, and infection.

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