SUMMARY
NIAID REGULATORY T CELL WORKSHOP
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Bethesda, MD

Definitions of T regulatory cells (Treg) are primarily functional, and some uncertainty continues over issues of surface marker expression and modes of suppressive activity. Nevertheless, the field is slowly piecing together the basic biology of Treg, with a greater appreciation of their influence on human immunity. The National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) convened a workshop on Treg in Bethesda, MD on November 20-21, 2006, during which researchers discussed the current state of affairs of Treg in both animal models and humans, covering tolerance in autoimmunity and allergy, transplantation immunology, and infectious disease.

The Rebirth of Treg

A number of mechanisms contribute to the capacity of the immune system to discriminate between self and non-self, facilitating the maintenance of immunological tolerance to self-antigens and the induction of protective immunity to foreign antigens. It is becoming clear that Treg play an important role in inducing self-tolerance and in preventing immune pathologies by limiting the strength and/or nature of the immune response to infectious pathogens.

Treg were intensely studied in the 1970s and early 1980s as suppressor T cells with a variety of functional characteristics. However, research on these cells stalled when definitive identification of the cells and effector molecules proved difficult. Then in 1995, Shimon Sakaguchi reported the identification of suppressor T cells by expression of the cell surface protein, CD25, as well CD4. CD25, the α-chain of the IL-2 receptor, was originally shown to be expressed on the surface of effector T cells, and has since been shown to be expressed on the surface of Treg. When Sakaguchi infused CD4+CD25- T cells into nude mice, recipients developed autoimmune disease. However, none was observed with the simultaneous adoptive transfer of CD4+CD25+ T cells. These experiments showed that a small, identifiable population of T cells worked to dampen autoimmune reactions. This work helped resurrect the field of Treg, and studies conducted in many animal models have confirmed the capacity of CD4+CD25+ Treg to prevent inflammatory pathologies *in vivo*. In addition, several other T cell subpopulations were identified that are capable of suppressive activity, resulting in renewed interest in the study of Treg and their implications in human disease.

Treg reduce the magnitude of immune responses by suppressing Th1 and/or Th2 cell function as well as that of other immune cells. Different Treg populations have been described based on origin, generation, and mechanism of action:

- 'natural' CD4+CD25+ Treg which develop in the thymus and regulate self-reactive T cells in the periphery;
- "induced" or "adaptive" CD4+CD25+ Treg that can be generated from mature CD4+CD25- T cells in the periphery after antigen stimulation;
- CD4+ Tr1 cells, which arise in the periphery and secrete IL-10 in the absence of IL-4;
- CD4+ Th3 cells, which are activated on the mucosal surface of the gut and secrete IL-10 and TGF-β; and
- CD8+ Treg.

CD4+CD25+ Treg

An overview of the field was presented by Shimon Sakaguchi at the NIAID Treg meeting, summarizing progress of the potential application of these cells in human disease. This overview addressed key issues regarding Treg and the induction of human disease. Evidence for the role of natural Treg in immune tolerance/immunoregulation in both rodents and humans was discussed. T cells that recognize autoantigens can be readily found in healthy individuals, but their activation is thought to be limited by Treg. There appears to be a clear dependence of the proliferation and survival of Treg on the presence of IL-2, as supported by Sakaguchi's most recent experiments demonstrating the induction of autoimmune disease in BALB/c mice by means of IL-2 neutralization. A surface molecule often found on natural Treg is GITR (glucocorticoid-induced tumor necrosis factor receptor). Sakaguchi found that inhibition of Treg by treatment with an anti-GITR monoclonal antibody could potentially be used for tumor eradication, although there is a caveat that autoimmunity might follow the systemic manipulation of Treg *in vivo*.

Foxp3: A good indicator?

The greatest challenge to fully understand the function and application of naturally occurring CD4+CD25+ Treg in humans is the lack of specific markers that define these cells and distinguish them from activated effector T cells and other Treg populations. Although the Foxp3 molecule has been suggested as an intracellular marker for naturally-occurring Treg, this is not as clear in humans. However, a recent observation by Maria-Grazia Roncarolo highlights the importance of Foxp3+ Treg in controlling human immunity. Patients carrying rare loss-of-function mutations in the *Foxp3* gene develop a range of autoimmune and inflammatory disorders referred to as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). The subsequent finding that Foxp3 is a transcription factor selectively expressed in and essential for the development of the CD4+CD25+ Treg lineage suggests that a defect in the naturally occurring Treg population underlies the clinical manifestations in IPEX patients. More importantly, it implies that the presence and efficient function of Treg is required to maintain health. However, although Foxp3 initially appeared to be exclusively expressed by CD4+CD25+ T cells with regulatory function, it is now known to be transiently up-regulated upon activation of naïve CD4+CD25- cell and CD8+ T cells in both mice and humans. Most human studies on CD4+ regulatory T cells use a combination of CD25,

cytotoxic T-lymphocyte-associated antigen (CTLA-4), Foxp3, IL-10 and/or TGF-β as markers in order to define Treg populations. Ultimately, only the demonstration of suppressive function confirms the presence of Treg.

Steven Ziegler presented related studies showing that the expression of Foxp3 in CD4+CD25- T cells results in the acquisition of a Treg-like phenotype in mice. To understand better the role of Foxp3 in T cell activation, he created transgenic mice that express Foxp3 in all CD4+ T cells to determine whether Foxp3 expression can mimic the effects on TCR signaling seen in Treg cells. These transgenic animals were generated using a genomic version of *Foxp3* as the transgene, and analysis of CD4+ T cell function in these animals revealed that all CD4+ T cells acquired Treg cell function. Both CD4+CD25- and CD4+CD25+ T cell subsets in Foxp3 transgenic (Tg) mice exhibit Treg activity when assessed *in vitro*. These findings suggest that Foxp3 is necessary for Treg function. Since both wild type (wt) CD4+CD25+ Treg cells and Foxp3 Tg CD4+ T cells display reduced responsiveness to TCR-mediated stimulation while inhibiting the response of effector T cells in an antigen-specific manner, T cells from Foxp3 Tg mice provide an opportunity to investigate the consequences of elevated Foxp3 levels on Treg function and T cell activation. In the most current studies from the Ziegler group, elevated levels of Foxp3 were found to increase Treg susceptibility to apoptosis following activation. In addition, Foxp3 Tg T cells were hindered in cell cycle progression following TCR engagement, possibly resulting in increased susceptibility to cell death. These data indicate that functionally and phenotypically, Foxp3 Treg cells can be equated with natural CD4+CD25+ Treg, and that these cells may have a dynamic life cycle with the flexibility to convert between Treg and effector T cells. Most recently, further complexity was added by evidence that cytokines within the microenvironment, such as TGF-β, can induce Foxp3+ Treg cells from CD4+CD25- precursors. This is the "induced" CD4+CD25+ Treg population. TGF-β-driven Treg were shown to inhibit innate inflammatory responses to syngeneic transplanted pancreatic islets in a murine model of transplantation. Understanding the signals necessary for the generation and expansion of Treg beyond the scope of Foxp3 expression will be important for clinical cellular therapy.

Tr1 Treg

Other Treg subsets, such as Tr1 Treg, may also play a role in targeting regulatory networks and the resolution of immunopathologies. Maria-Grazia Roncarolo reported on the role of IL-10 in the induction of peripheral tolerance and identified the IL-10-producing CD4+ T regulatory type 1 (Tr1) cells in humans. These studies emphasize the potential of targeting Tr1 cells to control/suppress pathogenic immune responses. *Ex vivo* or *in vivo* induction of Tr1 cells might be highly advantageous in several T cell-mediated diseases. In the allogeneic transplant setting, Roncarolo and colleagues demonstrated that donor PBMC cultured *ex vivo* with irradiated host PBMC in the presence of IL-10 become anergic towards host antigens while preserving the ability to proliferate in response to third party and nominal antigens.

CD8+ Treg

It is clear that in order to avoid pathogenic autoimmunity, peripheral regulatory mechanisms are required to limit the clonal expansion of potentially pathogenic self-reactive clones with TCRs whose affinity/avidity are not high enough to cause their intrathymic elimination. Although CD4+CD25+ Treg are though to play a role in self tolerance, peripheral tolerance mechanisms are also hypothesized to include regulatory CD8+ Treg. Leonard Chess and Hong Jiang have proposed mechanisms for CD8+ Treg-mediated regulation of the immune system involving the MHC Class Ib molecule, Qa-1. Qa-1 is the mouse homolog of human leukocyte antigen E (HLA-E) and forms a heterodimer with β2-microglobulin that binds to and presents peptides derived from self or foreign proteins. Its interaction with these types of ligands and the antigen receptor (TCR) on CD8 T cells is thought to inhibit natural killer (NK) cell activity and CD8 cytotoxic (CTL) activity. A characteristic feature of the Qa-1-dependent CD8+ T cells is that they require priming by activated CD4+ T cells during a primary immune response, and then can regulate secondary immune responses. This property distinguishes the CD8+ Treg from other cellular regulatory mechanisms, including NKT cells and CD4+CD25+ Treg, which exist predominantly as naturally occurring suppressor cells and function mainly during the early and primary phases of immunity.

Chess and Jiang have isolated these CD8+ Treg from mice and shown their ability to selectively down-regulate some selected myelin basic protein-reactive effector CD4+ T cells that are enriched as potentially encephalitogenic clones *in vivo*. The targets that are selectively down-regulated by these CD8+ Treg are not conclusively identified but recent work from Jiang and Chess suggests that the affinity/avidity of T cell effector clones might dictate their susceptibility to down-regulation by CD8+ Treg. To test this hypothesis and further delineate the biological functions of the CD8+ Treg in controlling peripheral immunity to both self and foreign antigens, they examined the regulation of mouse immune responses to a self antigen and a conventional antigen, hen egg lysozyme (HEL), and obtained evidence that Qa-1-dependent CD8+ Treg preferentially down-regulate effector T cells of intermediate affinity/avidity for both self and foreign antigens. To develop the therapeutic potential of CD8+ Treg, the precise role of Qa-1 in the regulatory pathway still needs to be defined.

Consistent with these results, Harvey Cantor demonstrated that Qa-1-deficient mice develop exaggerated secondary CD4 responses after viral infection or immunization with foreign and self peptides. This inhibitory CD8+ Treg:CD4 effector cell interaction may prevent expansion of pathogenic autoreactive CD4 T cell populations and consequent autoimmune disease and thus represent another population of T cell suppressor cells to target in human therapy. The delicate balance between allowing protective peripheral immunity and maintaining self tolerance is a threshold that has to be defined prior to the use of CD8+ Treg in treating human autoimmune disease. A central question remains concerning the properties of target CD4+ T cells that are susceptible to CD8+ Treg activity.

Treg and Infectious Disease

Significant challenges lie ahead for the treatment of dysregulated immunity in infectious disease and questions remain as to whether this can be accomplished by targeting natural Treg. Ethan Shevach and Yasmine Belkaid reported on the role of Treg in response to infection, particularly parasites and helminths.

Shevach addressed questions regarding the induction of subpopulations of Treg in the context of murine infection with *Leishmania major*, a model for studying chronic parasite-induced inflammation and Treg homing capacities. He demonstrated that Foxp3+ Treg can be subdivided into naïve-like and effector memory-like subsets based on surface expression of CD103 ($\alpha_E\beta_7$), which is found on 20-30% of CD4+CD25+ Treg following stimulation. Foxp3+CD103+ Treg are highly suppressive *in vitro,* and *in vivo* they migrate to inflamed tissues, such as the draining lymph nodes following subcutaneous inoculation with *Leishmania major*. Thymic medullary expression of MHC Class II appears to be required for the development of effector memory-like CD103+CD4+Foxp3+ T cells. CD103+ Treg cells also co-express NK cell-related markers and display a cell surface antigen profile resembling effector/memory T cells.

Thus, Foxp3+ Treg show evidence for a heterogeneity, involving both thymic-derived and well as thymic-independent populations. Preliminary studies suggest a therapeutic potential for these cells and their migration to inflamed tissues in the initial stages of the response to infection may represent a key defense mechanism to be exploited.

It is clear that a consequence of the modulation of excessive immune responses by natural Treg might be enhanced pathogen survival and long-term persistence. C57BL/6 mice infected with a low dose of *Leishmania major* develop small self-healing lesions, and the immunity to re-infection that is seen in other mouse strains requires a persistent infection. Thus, Treg can promote persistence and potential transmission to other hosts. Yasmine Belkaid demonstrated that CD4+CD25+ Treg can respond specifically to foreign antigens: they strongly proliferate in response to *Leishmania*-infected dendritic cells; they maintain Foxp3 expression; and *Leishmania*-specific Treg cell lines can be generated from infected mice. The majority of such Treg at the infected site are *Leishmania* specific; parasite-specific Treg are restricted to sites of infection and their survival is strictly dependent on parasite persistence. The vast majority of Treg present in the dermis at steady-state conditions or during *Leishmania* infection express the α_E chain of the integrin CD103 consistent with the Shevach results. Demonstration of Ag-specific Treg homing in the context of infection presents a window of opportunity for therapeutic applications. Additionally, models of infection should be studied to help define new treatments for infection in both acute and chronic diseases.

Treg and Autoimmune Disease

Human CD4+CD25+ natural Treg have *in vitro* features similar to the rodent CD4+CD25+ T cell population. As David Hafler summarized at the meeting, adult humans are exposed to a wide range of microbial infections, and simply gating on the CD4+CD25+ T cells appears to capture both Treg and activated effector T cells. To

identify additional markers for Treg, he presented findings which support the existence of a subtype of human CD4+CD25+ cells that are HLA DR-positive. These DR+ Treg isolated from human peripheral blood samples express significantly higher levels of Foxp3 message and protein than HLA DR- Treg. Suppression by the DR+ Treg is contact-dependent, whereas DR- CD4+CD25+ Treg induce early IL-4 and IL-10 secretion and late Foxp3-associated contact-dependent suppression. Direct single-cell cloning of CD4+CD25+ Treg revealed that, regardless of initial DR expression, *ex vivo* expression of CD25, and not DR, predicts which clones will exhibit contact-dependent suppression, high levels of Foxp3 message, and an increased propensity to become constitutive for DR expression. DR expression appears to define a distinct subpopulation of Treg cells.

Other studies by Hafler described Treg defects in patients with multiple sclerosis (MS), an inflammatory disease of the central nervous system. The function of CD4+CD25+ Treg derived from MS patients was decreased compared with control subjects, although the frequency of CD4+CD25+ T cells was the same between the two populations. Differences were also apparent in single-cell cloning experiments, as the cloning frequency of CD4+CD25+ T cells was significantly reduced in patients compared with normal control subjects. These assays demonstrated that Treg isolated from the circulation of patients with MS exhibited defects in Treg function and clonal expansion. Whether these Treg have differences with regard to DR expression is unknown. More recently, Hafler also reported a deficit in CD46-mediated induction of Tr1 Treg in patients with MS. CD46 is a newly identified co-stimulatory molecule for T cell activation, and CD46 co-stimulated human T cells have a Tr1 Treg phenotype with considerable amounts of IL-10 secretion. These data demonstrate a second major Treg defect in a human autoimmune disease, associated with the CD46 pathway and TR1 cells.

Treg and Transplantation

Maria-Grazia Roncarolo reported that CD4+CD25+ Treg can be expanded *in vitro* by the combination of anti-CD3 antibody, anti-CD28 antibody and rapamycin. These expanded cells show an increase in Foxp3 expression. Rapamycin is an immunosuppressive compound that is currently used to prevent acute graft rejection in humans. In addition, rapamycin has been shown to allow operational tolerance in murine models. However, a direct effect of rapamycin on Treg has not yet been demonstrated. Roncarolo reported that rapamycin selectively expands murine naturally occurring CD4+CD25+Foxp3+ Treg *in vitro*. These expanded Treg suppress proliferation of syngeneic T cells *in vitro* and prevent allograft rejection *in vivo*. She also demonstrated that activation of human CD4+ T cells from healthy subjects in the presence of rapamycin leads to growth of CD4+CD25+Foxp3+ Treg and selective depletion of CD4+CD25- T effector cells. The rapamycin-expanded induced Treg suppress proliferation of both syngeneic and allogeneic CD4+ and CD8+ T cells. Interestingly, rapamycin does promote expansion of functional CD4+CD25+Foxp3+ Treg in cells from type 1 diabetic patients, reported to have defects in freshly isolated CD4+CD25+ Treg. These data indicate that selected immunomodulatory compounds might induce Treg *in vivo*, and the capacity of rapamycin to allow growth of functional CD4+CD25+Foxp3+ Treg, and also to deplete T effector

cells, might be exploited for the design of novel and safe protocols for cellular immunotherapy in T cell-mediated diseases.

T cell-depleting agents are being tested as part of clinical tolerance strategies in humans with autoimmune disease or in transplantation. Mohamed Sayegh reported on studies using polyclonal anti-thymocyte globulin (ATG), the purified IgG fraction of sera from rabbits, horses, or more rarely, goats that are immunized with human thymocytes or T cell lines. ATG is used for treatment of various clinical conditions, including prevention or rescue treatment of acute rejection in organ transplantation, conditioning for hematopoietic stem cell transplantation, treatment of severe aplastic anemia, various autoimmune diseases, and more recently, graft-versus-host disease. Sayegh reports that the immunosuppressive activity of ATG has been thought to result primarily from the depletion of peripheral lymphocytes from the circulating pool through complement-dependent lysis or activation-associated apoptosis. He was the first to report that ATG, but not anti-CD52 or IL-2R antagonists, causes rapid and sustained expansion of CD4+CD25+ Treg when cultured with human peripheral blood lymphocytes. These Treg display enhanced expression of the markers glucocorticoid-induced TNF receptor (GITR), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and Foxp3 and efficiently suppress a direct alloimmune response of the original responder lymphocytes. ATG-induced expansion of Treg was mainly due to conversion of CD4+CD25- into CD4+CD25+ T cells and to a lesser degree to proliferation of natural CD4+CD25+ T cells. Thus, the use of ATG seems a promising option for Treg activation in allograft tolerance. These data suggest that ATG not only may promote expansion/generation of Treg but also may be useful in future *in vitro* expansion of these cells for cellular therapy in autoimmunity and clinical transplantation. Clinical applications may be limited due to unknown, long-term bystander effects of ATG which have yet to be determined.

Treg and Allergic Disease

One key example of the potential for Treg and clinical applications lies within the field of allergic disease. Recent modified approaches to allergen immunotherapy include shorter and pre-seasonal regimens and alternative routes of administration, particularly the sublingual route. Immunotherapy can be currently prescribed for patients with IgE-dependent disease and is particularly effective in patients with insect venom anaphylaxis and in those with severe seasonal allergic rhinitis unresponsive to anti-allergic drugs. Although these current treatments can be effective, the targeted cell populations and the long-term benefits are unknown. Stephen Durham described a CD4+CD25+ Treg population in PBMC from atopic *versus* non-atopic donors which may provide some insight. His data highlight house dust mite (HDM) immunotherapy and the induction of Treg that consistently produce IL-10 and TGF-β after patients are treated with allergen immunotherapy *versus* those who received placebo. This increase in IL-10 and TGF-β following allergen immunotherapy was detectable not only in peripheral blood T cells but also in the nasal mucosa, and increased allergen-specific IgG4 antibody was also seen. Overall, subcutaneous immunotherapy in individuals allergic to HDM is highly effective and correlates with the induction of IL-10/TGFβ-producing Treg. Future clinical applications might target these cells for optimal prevention of allergic reactions. Thus

far, the issue of allergen specificity for sensitized individuals has not been addressed and the precise relationship between natural and adaptive subsets of Treg is not yet defined.

Dale Umetsu reported on recent studies showing that soluble peptide-class II MHC tetramers can be used to directly identify and study allergen epitope-specific CD4+ T cells in the hope of addressing the issue of antigen specific Treg. In previous work, he demonstrated the detection of antigen specific Treg in a murine model of OVA-induced airway inflammation. More recently, using DRB1*0401 tetramers loaded with the major epitope of the rye grass allergen, *Lol p 1*, he detected allergen-specific CD4+ T cells in the peripheral blood of DRB1*0401 rye grass allergic individuals after *ex vivo* expansion with allergen. These tetramer-positive cells produced IL-4, but little IFN-γ. In contrast, rye grass tetramer-positive cells were not detected in cultures from HLA-DR*0401 non-allergic patients, even after expansion with IL-2. These recent results suggest that rye grass allergen-specific T cells in DR*0401 non-allergic subjects are present at very low levels (due to deletion or suppression), differ in a fundamental way in their requirement for *ex vivo* expansion, or use TCRs distinct from those of allergic patients. The current technology does not yet allow for detection of rye grass pollen-specific Treg.

On the horizon?

There are still many questions regarding the clinical use of Treg for the cure of T cell-mediated diseases and overall immune-driven pathology. Many of the questions which were set forward more than a decade ago in this field have been answered: Treg isolation, cytokine secretion profiles, initial cell-surface markers. However, the large body of information provided by *in vitro* studies and by studies dissecting the role of Treg in different disease models has also generated new questions and some degree of confusion. Results from *in vivo* studies have not always been consistent with *in vitro* observations. This disparity is due not only to the lack of completely exclusive markers and the inadequacy of CD25 as a specific marker for natural Treg, but also to the undefined mechanisms of suppression by CD4+CD25+ Treg and other Treg subtypes and the paucity of studies in which Treg are characterized at the clonal level. Based on this meeting's content, it is clear that the field of Treg has advanced significantly. Not only are there compelling data to increase the prospect of using these cells to treat various diseases in animal models, but also convincing data in human studies. Despite these advances, questions of safety and non-specific activation of bystander cells remain. Furthermore, Treg may express different markers based on migratory patterns, such as the CD103 example. Targeting Treg for therapy may require a more tailored approach than we currently appreciate, that is disease-specific. However, it is clear that the use of Treg in the clinic for the treatment of human diseases is rapidly becoming a more plausible form of future therapy.

*Workshop Participants: Shimon Sakaguchi, Kyoto University; Leonard Chess, Columbia University; David Hafler, Harvard Medical School; Maria-Grazia Roncarolo, San Raffaele Telethon Institute for Gene Therapy; Steven Ziegler, Benaroya Research Institute; Ethan Shevach, NIAID; Yasmine Belkaid, NIAID; Mohammed Sayegh, Brigham and Women's Hospital; Stephen Durham, Imperial College, London; Dale

Umetsu, Children's Hospital of Boston; Carl June, University of Pennsylvania; James Markmann, University of Pennsylvania; Harvey Cantor, Dana-Farber Cancer Institute; Hong Jiang, Columbia University; Barry Rouse, University of Tennessee; Arlene Sharpe, Harvard Medical School; Andrew Mellor, Medical College of Georgia.