Regulatory T cells (Tregs) constitute a subset of lymphocytes that have the capability of suppressing immune responses in vivo and in vitro both directly by cell-cell contact and indirectly through the production of anti-inflammatory cytokines, such as interleukin-10 and tumor growth factor-β. Tregs constitute a small subset of T lymphocytes, yet their presence can prevent and control autoimmune disease and organ transplant rejection and contribute to maternal tolerance of fetal alloantigens, whereas their absence results in uncontrolled inflammation. But Treg function may not always be considered beneficial: There is growing evidence that the immunosuppressive effects of Tregs are also associated with growth of tumor cells. Thus, Tregs are of considerable medical interest as targets for the treatment of both inflammatory diseases and cancer. In this review of published literature, we describe some well-characterized immunomodulatory drugs and environmental toxicants that can either positively or negatively affect the number and/or function of Tregs in animal models and/or human patients. The targeted suppression or enhancement of Treg function needs to be carefully considered in immunotoxicology evaluations as manipulation of this immune cell subset of T lymphocytes, yet their presence can prevent and control autoimmune disease and organ transplant rejection and contribute to maternal tolerance of fetal alloantigens, whereas their absence results in uncontrolled inflammation. But Treg function may not always be considered beneficial: There is growing evidence that the immunosuppressive effects of Tregs are also associated with growth of tumor cells. Thus, Tregs are of considerable medical interest as targets for the treatment of both inflammatory diseases and cancer. In this review of published literature, we describe some well-characterized immunomodulatory drugs and environmental toxicants that can either positively or negatively affect the number and/or function of Tregs in animal models and/or human patients. The targeted suppression or enhancement of Treg function needs to be carefully considered in immunotoxicology evaluations as manipulation of this immune cell population could result in undesired consequences, including decreased host resistance, decreased fertility, or increased incidence of inflammatory disease.

Key Words: regulatory T cells; immunomodulatory drugs; FoxP3; immunotoxicology.

The natural function of the immune system is to respond to bacterial, viral, fungal, rickettsial, and parasitic invaders of the host by coordinating an effort among multiple immune cell types to eliminate or control the intruders. These immune responses are critical for the host’s survival, as is made clear by an increased incidence of infection, often leading to death, in individuals lacking functional components of the immune response. Just as important as mounting an immune response, organisms have checks and balances to control the magnitude and duration of the immune response. Uncontrolled inflammation can lead to serious damage to one or multiple organs, such as in autoimmune diseases, or even death, such as in septic shock or a “cytokine storm.”

The mammalian immune system has evolved to allow for the development of suppressor immune cells: a particular subset of immune cells that work through various mechanisms to tone down or eliminate inflammatory responses to pathogens and to environmental antigens, such as food antigens and commensal bacteria. Immune cells with immunosuppressive function include a subset of CD8+ T cells (Filaci et al., 2007), myeloid suppressor cells (Ostrand-Rosenberg and Sinha, 2009), and a subset of CD4+ T cells (Bach, 2003). Of all the immune cells with suppressor function, the best understood are the CD4+ suppressive T cells also known as regulatory T cells (Tregs). Two main types of Tregs have been described in the literature. Natural Tregs, which leave the thymus as functional major histocompatibility class II–restricted suppressors cells, are essential for the prevention of autoimmunity and keeping immune responses to pathogens under control (Bluestone and Abbas, 2003; Ito et al., 2008; Sakaguchi et al., 2006). Natural Tregs are characterized by constitutive cell surface expression of high levels of the interleukin (IL)-2 receptor alpha chain and cytotoxic T-lymphocyte antigen 4 (CTLA-4); expression of the forkhead box P3 (FoxP3) transcription factor in the nucleus; low levels of the interleukin (IL)-2 receptor alpha chain and tumor growth factor–β (TGF-β); and their ability to inhibit effector T-cell (Teff) proliferation and release of interferon-γ (IFN-γ), tumor necrosis factor–α (TNF-α), and other proinflammatory cytokines in vitro in response to antigenic or mitogenic stimulation (Fig. 1). Adaptive or induced Tregs are antigen-stimulated CD4+ T cells that develop into functional suppressors in the presence of IL-10 (Tr1 cells) or TGFβ (T helper 3 cells) in the periphery and thus contribute to the development of an antigen-specific immunosuppressive response (Bluestone and Abbas, 2003; Roncarolo et al., 2006).
Such adaptive Tregs can also be FoxP3\(^+\) and can closely resemble natural Tregs in function.

**EVIDENCE FOR THE IMPORTANCE OF Treg FUNCTION IN MICE AND HUMANS**

Evidence, both in mice and in humans, clearly shows the importance of Treg function to prevent chronic multiorgan inflammation. The expression of the FoxP3 transcription factor is required for Tregs to function as immunosuppressive cells (Fontenot \textit{et al.}, 2003). A spontaneous mutant strain of mouse, known as scurfy mice, is known to develop a fatal lymphoproliferative disorder, characterized by runting; scaliness and crusting of the eyelids, ears, and tail; marked splenomegaly; enlarged lymph nodes; and severe anemia. In 2001, the mutation in the scurfy mice was determined to be a disruption in the \textit{foxp3} gene, and the transfer of wild-type Tregs to newborn scurfy mice could prevent the development of the lymphoproliferative disorder (Brunkow \textit{et al.}, 2001). This chronic inflammatory phenotype is similar for FoxP3 knockout mice (Fontenot \textit{et al.}, 2003). Two research groups have developed mouse strains with an inducible disruption of \textit{foxp3} gene function such that exposure to diphtheria toxin resulted in elimination of FoxP3-expressing cells. In one laboratory, adult animals without functional Tregs developed stronger delayed-type hypersensitivity responses than wild-type mice. Interestingly, these Treg-depleted adult mice were not described as developing a lymphoproliferative disorder, at least for the duration of the study, whereas disruption of Treg function during the newborn phase did lead to a lymphoproliferative disorder almost identical to the scurfy mice (Lahl \textit{et al.}, 2007). However, in a separate study in Treg-depleted adult mice, animals developed severe lymphadenopathy, splenomegaly, severe conjunctivitis, weight loss, and died within 3 weeks of elimination of FoxP3-expressing cells after exposure to diphtheria toxin (Kim \textit{et al.}, 2007).

Why depletion of FoxP3\(^+\) Treg resulted in uncontrolled activation of the immune system in one set of adult mice, but not another set, is not clear. Several factors, including differences in how the mutant animals were created, housing conditions, and the types of commensal bacteria present in the animal colony, could contribute to the differences in outcomes. Regardless, these data indicate that it may be difficult to predict the effects of Treg depletion in adult humans.

In humans, two chronic inflammatory diseases have been linked to a decrease in Treg function. Immunodysregulation...
polyendocrinopathy enteropathy X linked (IPEX) is a rare disorder characterized by dermatitis, cachexia, growth retardation, autoimmune enteropathy, type 1 diabetes mellitus, and chronic inflammation with excessive cytokine production, which can lead to thyroiditis, autoimmune hemolytic anemia, recurrent infections, and membranous nephropathy. Like the scurfy mice, IPEX patients have a missense mutation in their foxp3 gene in the DNA binding domain or have a mutation outside of the foxp3 gene, which affects FoxP3 function (van der Vliet and Nieuwenhuis, 2007). Treatment for IPEX patients includes immunosuppressive therapy and bone marrow transplantation. Autoimmune polyendocrinopathy-candidiasis-ectodermal dysplasia (APECED; also known as autoimmune polyendocrine syndrome I) is a rare monogenic recessive disease in humans that is characterized by inflammation in multiple tissues, including hypoparathyroidism, Addison’s disease, and type 1 diabetes mellitus. APECED patients have a loss-of-function mutation in the “autoimmune regulator” (AIRE). Interestingly, AIRE-deficient mice display signs of immune system dysregulation but remain clinically healthy. Kekalainen et al. (2007) have shown that APECED patients have decreased FoxP3 expression compared with healthy controls, yet AIRE-deficient mice have normal FoxP3 expression. These data suggest that APECED patients have a deficiency in Treg function, which AIRE-deficient mice do not have, and indicate that there are genes other than foxp3 involved in the suppression of the immune response.

Both IPEX and APECED are caused by genetic mutations present from conception, similar to the scurfy mutation in mice. The effect of the absence of Tregs in adult humans is less well documented. However, there is some evidence for decreased numbers and/or function of Tregs in several autoimmune diseases. A decrease in suppressive function in the Treg population in the peripheral blood of multiple sclerosis patients compared with healthy controls has been documented (Viglietta et al., 2004). Furthermore, there is negative correlation between the number of circulating Tregs and serum levels of anti-dsDNA antibody in patients with systemic lupus erythematosus (SLE) (Lee et al., 2008). These data suggest that a lack of Treg function may correlate with the development of autoimmune disease even in adults, though it is difficult to determine if the decreased Treg function is a cause or an effect of the chronic inflammatory state.

The ability of Tregs to suppress inflammatory responses is both a desired and an undesired function in different disease states. For cancer, there is growing evidence that the presence of Tregs and/or other suppressive immune cells prevents the immune system from rejecting tumor antigen–bearing cells and thus aid the continued growth and spread of the cancer cells (Colombo and Piconese, 2007; von Herrath and Homann, 2003). In this setting, the actual or functional elimination of Tregs is undesirable. For autoimmune diseases and for the prevention of organ transplant rejection, increased numbers and/or effectiveness of Tregs was the goal (von Herrath and Homann, 2003). Therefore, manipulating the Treg compartment, both in size and in activity, has become an attractive strategy in the control of immunopathology (Gogishvili et al., 2009).

AUGMENTING Treg FUNCTION

Immunosuppressive Agents Known to Increase Treg Numbers and/or Function: Rapamycin, Corticosteroids, Antithymocyte Globulin, Alemtuzumab, Statins, and AhR Ligands

The enhancement of Treg function, including the transfer of isolated Tregs, is a therapeutic goal for the treatment of autoimmune disease (Roncarolo and Levings, 2000; Trzonkowski et al., 2009; von Herrath and Homann, 2003). Several marketed therapeutics are known to favor the expansion of Tregs over Teffs (see Table 1). Rapamycin (sirolimus), a macrolide antibiotic, is routinely used as an immunosuppressive drug in recipients of organ transplants. Within the past 5 years, it has been discovered that rapamycin and the rapamycin derivative everolimus selectively induce the apoptosis of murine and human Tregs but not Tregs in vitro and even allow for the preferential expansion of Tregs in vivo (Battaglia et al., 2005, 2006; Coenen et al., 2006; Gane et al., 2005). Furthermore, in a murine model of acute graft versus host disease (GVHD), cotreatment of animals with both isolated Tregs and rapamycin was more effective than either treatment alone in preventing death because of T-cell–based rejection of bone marrow transplant (Zeiser et al., 2008). The mechanism by which rapamycin selectively induces apoptosis in Teffs but not Tregs is still under investigation, but preliminary evidence indicates that expression of PTEN and/or Pim2 in Tregs protects these cells from rapamycin-induced inhibition of the mammalian target of rapamycin (mTOR) signaling pathway (Basu et al., 2008; Zeiser et al., 2008), suggesting that it may be possible to develop other therapeutics that specifically target Teffs or Tregs.

Corticosteroids are another class of drugs that have been used for decades to suppress inflammatory reactions and treat autoimmune diseases. Recent evidence suggests that, like rapamycin, some of the immunosuppressive effects of corticosteroids may be because of a selective sparing of Tregs in comparison with Teff. Murine Tregs are more resistant than Teffs to the apoptotic effects of dexamethasone and treatment of mice with dexamethasone results in an increased proportion of Tregs in the lymphoid organs (Chen et al., 2004). In humans, treatment of asthmatic patients with either inhaled or systemic glucocorticoids resulted in increased FoxP3 and IL-10 messenger RNA expression and an increased proportion of circulating Tregs (Karagiannidis et al., 2004). Furthermore, SLE patients treated with glucocorticoids either alone or in combination with other therapies had a higher proportion of circulating Tregs than patients on other immunosuppressive drugs or normal healthy volunteers (Suarez et al., 2006).
Immune suppressive effects positively correlates with generation of CD25+ CD4+ T cells in rodents; TCDD-derived CD4+ T cells produce IL-10 but not IL-2 and show contact-dependent suppressive function.

Other immunosuppressive therapies are also showing positive effects on Treg numbers and/or function. Polyclonal antithymocyte globulin (ATG) is a purified IgG fraction whose mechanism of action is the depletion of T cells by either complement-dependent lysis or activation-induced apoptosis. ATG is currently being used for the prevention or rescue of acute rejection of organ transplants, conditioning for hematopoietic stem cell transplantation, treatment of severe aplastic anemia, and various autoimmune diseases. Recently, Lopez et al. (2006) have shown that exposure of human peripheral blood lymphocytes ex vivo to ATG results in expansion of Tregs within 24 h and can induce development of Tregs from Teff. These data have been confirmed by another group that went on to show that this effect is specific for ATG derived from rabbits but not ATG derived from horses (Feng et al., 2008). The mechanism by which rabbit-derived ATG favors the development of adaptive Tregs remains to be elucidated. Alemtuzumab (Campath-1H) is a monoclonal humanized antibody specific for CD52, a small glycopeptide found on the cell surface of lymphocytes; treatment of transplant recipients with alemtuzumab results in depletion of circulating T cells, B cells, and other lymphoid subsets (Ciancio et al., 2005). Recently, it has been discovered that costimulation of human T cells in vitro with alemtuzumab plus an anti-CD3 mAb results in the preferential expansion of Tregs, though the mechanism is currently unknown (Watanabe et al., 2006; Basu et al., 2008). These in vitro results that indicate that ATG and alemtuzumab can aid in expansion of Tregs correlate with the observation that renal transplant patients treated with alemtuzumab or with ATG have increased proportions of circulating Tregs (Ciancio et al., 2005).

Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMG-CoA), or statins, are in widespread use because of their cholesterol-reducing properties and concomitant improvement of clinical outcome in patients with and without preexisting atherosclerosis. There is growing evidence that Tregs play a beneficial role in protecting against atherosclerosis in animal models and that patients with acute coronary syndrome have...
defects in Treg numbers and/or function (Ait-Oufella et al., 2006) (Mor et al., 2006, 2007). Therefore, the ability of statins to affect Treg numbers and function is currently under investigation. In one study, patients treated with pravastatin and simvastatin showed increased proportions of circulating Tregs after 4 and 8 weeks of treatment. Furthermore, exposure to atorvastatin in vitro induced human Teffs (CD4+CD25+FoxP3−) to become adaptive Tregs (CD4+CD25+FoxP3+) (Mausner-Fainberg et al., 2008). In spite of the beneficial effects of statins, concerns have been raised about the long-term immunomodulatory risks through prolonged use of statins. Goldstein et al. (2008) attempted to link the increase in circulating Tregs in statin-treated patients to explain the inverse correlation between achieved low density lipoproteins cholesterol levels and risk of cancer shown in a recent prospective analysis of clinical trials using statins. However, all these associations are speculations, given our current state of knowledge.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an AhR ligand that induces immune suppression in animal models, resulting in decreased T-dependent antibody responses, increased susceptibility to infectious disease, and prevention of rejection of tumor transplants. T lymphocytes, including both CD4+ T helper and CD8+ T-cytotoxic cell functions, are known to be suppressed in vivo in the presence of TCDD (Kerkvliet et al., 2002). It has been shown recently that the immunosuppressive effect of TCDD exposure in mice correlated with the generation of CD25+CD4+ T cells with suppressive function (Funatake et al., 2005) (Marshall et al., 2008) (Kerkvliet et al., 2009). Accordingly, Marshall et al. (2008) performed a study to characterize the TCDD-derived CD4+ T cells in terms of cellular anergy, suppressive functions, and cytokine production. TCDD-derived CD4+ T cells actively proliferated in response to various stimuli but suppressed IL-2 production and proliferation of Teffs. Like natural Tregs, TCDD-derived CD4+ T cells did not produce IL-2 and their suppressive function was contact dependent. TCDD-CD4+ cells also tended to secrete significant amounts of IL-10 in response to both polyclonal and alloantigen stimuli. Interestingly, only 2% of TCDD-derived CD4+ T cells express FoxP3, suggesting that the AhR does not rely on FoxP3 for its suppressive activity (Marshall et al., 2008). These data contrast with that of another group that showed that exposure to TCDD resulted in an increased proportion of murine FoxP3+ Tregs both in vitro and in vivo and that these Tregs suppressed immune responses through a TGFβ−, but not IL-10−, dependent mechanism (Quintana et al., 2008). Interestingly, in the same study, the exposure of murine immune cells to another AhR ligand 6 formylindolo[3,2-b] carbazole (FICZ) in combination with TGFβ resulted in the formation of proinflammatory IL-17-secreting T cells that could exacerbate inflammation in vivo. Therefore, the role of AhR in immunosuppression versus inflammation requires further investigation. Though there is clearly evidence that TCDD can induce the formation of Tregs, it can also negatively impact the function of several immune cells types. Therefore, it remains to be determined if the generation of IL-10 or TGFβ-producing CD4+ T cells is a major component of the immunosuppressive state of TCDD-treated animals.

### Novel Mechanisms for Augmentation of Treg Function

The use of IL-2 to preferentially expand Tregs is currently being explored. IL-2 is a necessary signal for the development, survival, and expansion of Tregs (Oppenheim, 2007) (Malek and Bayer, 2004). The cotreatment of mice with dexamethasone and recombinant murine IL-2 led to the selective expansion of Tregs in the spleen and lymph nodes, and cotreatment of mice with dexamethasone and IL-2 complementary DNA resulted in resistance to the development of experimental autoimmune encephalomyelitis (Chen et al., 2006). However, the use of IL-2 to aid immunosuppressive therapies in humans remains to be tested. As another method aimed at expansion of Tregs is superagonistic CD28-specific monoclonal antibodies, which were developed to cause polyclonal Treg activation to return activated immune cells to a quiescent state (Hunig and Dennehay, 2005) (Hunig, 2007). A superagonist CD28-specific mAb (CD28SA) has been used in various rodent models for Treg-based interference with autoimmunity and inflammatory disease models. Beyersdorf et al. (2005) demonstrated that a rat CD28SA was highly effective in vivo in expanding the size and enhancing the activity of the Treg compartment, leading to substantial therapeutic success in rat models of autoimmunity and inflammation (Beyersdorf et al., 2005). However, in contrast to the beneficial effects demonstrated in the rat, a fully humanized human CD28SA, TGN1412, induced a life-threatening cytokine release syndrome during a first in human trial (Suntharalingam et al., 2006). This raises questions specifically about the relationship between the induction of toxic cytokine release by the CD28SA on one side and their ability to mediate the desired effect of polyclonal Treg activation on the other. To further elaborate the role between Treg activation and systemic cytokine release, Gogishvili et al. (2009) used a novel anti-mouse CD28SA. Treg activation by this anti-mouse CD28SA was highly efficient but depended on paracrine IL-2 from CD28SA-stimulated conventional T cells. Systemic cytokine levels were relatively low, but depletion of Tregs prior to CD28SA stimulation led to systemic release of proinflammatory cytokines (TNF-α, IL-6, and IL-2). However, this still leaves a gap in our understanding of why TNG1412 behaved very differently in humans compared with the behavior of the rodent-specific CD28SA in rats and mice.

### Increasing Treg Numbers and/or Effectiveness: Concerns for Host Resistance to Infections?

The major concern with all immunosuppressive therapies is that the patient will have increased susceptibility to infection,
including potentially lethal infections. The role of Tregs in infectious disease is complex. On the one hand, Tregs play an important role in controlling immune responses to infectious agents as to prevent damage to surrounding tissues, as has been demonstrated with animal models of herpes simplex virus and *Schistosoma mansoni* infection. On the other hand, there is growing evidence that Treg function during infection can prevent clearance and/or lead to long-term survival of the infectious organism, including hepatitis B and C viruses (human), *Leishmania major* (mice), and *Plasmodium yoelii* (mice) (reviewed in Belkaid, 2007) (Joosten and Ottenhoff, 2008). Another example of the delicate balance of Treg function in the immune response to infection is found in a recent study on human interethnic susceptibility to malarial 

*Plasmodium falciparum* infection. Members of the Fulani ethnic group in West Africa have a lower incidence of malarial infection compared with another ethnic group (Mossi) in the same region, despite similar exposure rates to similar strains of the parasite. An investigation by Torcia et al. (2008) showed that the strong immune response to malarial parasites seen in the Fulani correlated with an increased expression of T helper 1–related genes and a reduced expression of Treg-related genes, particularly CTLA4 and FoxP3, and TGFβ. Furthermore, they showed that removal of Tregs from peripheral blood mononuclear cells (PBMCs) obtained from Mossi volunteers increased the ability of their immune cells to respond to malarial antigens in vitro. Interestingly, this same ethnic group, although relatively protected from malarial infection, also has a higher susceptibility to autoimmune disease, including type 1 diabetes, pemphigus, and onchocercal skin disease (Torcia et al., 2008), reinforcing the concept that the optimal balance of Tregs function may be difficult to predict in humans.

**DIMINUTION OF TREG FUNCTION**

*Immunosuppressive Agents Known to Negatively Affect TREG Function: Cyclophosphamide and Calcineurin Inhibitors*

As Tregs are lymphoid cells, they have many cellular mechanisms in common with other lymphoid cell types. Thus, it is not surprising that there are already therapeutic compounds being used in patients for various diseases, which have either positive or negative effects on Tregs, and these examples can be instructive of the effects of the modulation of Treg function in patients (summarized in Table 1).

Cyclophosphamide (CTX) is a cytotoxic alkylating nitrogen mustard compound that was developed as a chemotherapeutic agent for treatment of cancer but in addition has selective toxicity on lymphoid, but not myeloid, cells. CTX can be used as an immunosuppressive or immunostimulatory (“augmentory”) drug, depending on the timing and the dose when the drug is given in relation to antigen stimulation. High doses of CTX are cytotoxic to all lymphocytes and are used to prevent GVHD, SLE, and advanced rheumatoid arthritis (Brode and Cooke, 2008). However, Berd et al. (1982) showed that treatment of patients with low doses of CTX resulted in increased delayed-type hypersensitivity responses to keyhole limpet hemocyanin, if the drug was given prior to vaccination with the antigen. More recently, Ghiringhelli et al. (2004, 2007) have shown both in a rat tumor model and in human cancer patients that treatment with iterative low CTX doses (1/3 to 1/10 of the maximum) resulted in a decreased number of Tregs in the spleen (rat) and peripheral blood (human) without affecting other lymphocyte subsets. Furthermore, PBMC isolated from the CTX-treated human patients had increased natural killer (NK) cell–mediated killing activity and an increased proliferation response to anti-CD3/anti-CD28 stimulation in vitro compared with before treatment began (Ghiringhelli et al., 2007). Treatment with CTX has been shown not only to cause a decrease in the number of Tregs in rodents but also to accelerate the development of experimental autoimmunity, including type 1 diabetes (non-obese diabetic mice), experimental autoimmune encephalitis, and allergic inflammatory airway disease (Brode and Cooke, 2008). The mechanism by which Tregs are more susceptible to the cytotoxic effects of CTX is unclear. Tregs may be intrinsically more susceptible to the direct cytotoxic effects of CTX or CTX may act indirectly on Tregs by inhibiting IL-2 production at low doses without directly killing Teffs. Tregs are exquisitely sensitive to IL-2 concentrations and their survival and function depend on IL-2 availability (Malek and Bayer, 2004).

Because of this strong dependence on IL-2, Treg function is inhibited in the presence of calcineurin inhibitors (CNI), such as cyclosporine. Zeiser et al. (2006) have shown using a mouse model of acute GVHD that the presence of Tregs is beneficial for the prevention of acute GVHD but that the cotreatment with cyclosporine A (CsA) inhibited the protective effect of the Tregs. This inhibitory effect of CsA on Tregs in this GVHD model was not seen with either rapamycin or mycophenolate mofetil and could be overcome with the addition of exogenous IL-2. Furthermore, Segundo et al. (2006) have documented a decreased proportion of peripheral Tregs in kidney transplant patients treated with CNI compared with rapamycin-treated transplant patients or healthy controls. And they were able to correlate an accelerated decrease in kidney function over time in CNI-treated transplant patients versus rapamycin-treated transplant patients, suggesting that decreased Treg function could potentially increase transplant rejection.

**Novel Mechanisms for the Inhibition of TREG Function**

The C-C chemokine receptor 4 (CCR4) has been posited as a target for the inhibition of Treg function based on its expression on ≥ 90% of human Tregs as well as a majority of helper T cells associated with allergic responses (Th2). CCR4 antagonists coadministered with vaccine antigens demonstrate enhanced dendritic cell-mediated human CD4+ T-cell proliferation in vitro and amplified cellular and humoral immune responses after vaccination in vivo in mice (Bayry et al., 2008).
These data suggest that CCR4 antagonists may inhibit Treg function and exert adjuvant activity in vaccination in animal models.

Beyond being amenable to drug-induced modulation, Tregs may also be impacted by environmental contaminants. Arsenic (As) is an element of immunological and medical interest because it is apparent that it affects various functions as well as survival of immune cells and because a positive therapeutic effect of As in acute promyelocytic leukemia has been demonstrated. Hernandez-Castro et al. (2009) investigated the effects of As on Tregs and demonstrated that low concentrations of As tend to increase the number of Tregs (CD4⁺CD25⁺Foxp3⁺CTLA-4⁺) in human peripheral blood mononuclear cells in vitro, whereas higher concentrations had an opposite effect. The authors considered this an interesting finding because it has been suggested that As may have therapeutic potential for the treatment of autoimmune diseases. Furthermore, assessing different subsets of T lymphocytes in PBMC from individuals exposed to As indicated a highly significant negative correlation between the numbers of circulating Tregs and the levels of urinary As. The negative effect of high doses of As on Treg numbers seen in vitro and in vivo could be because of the proapoptotic effects of As on lymphocytes. The mechanism behind the increased numbers of Tregs at low doses of As remains speculative but may indicate a beneficial role for this metalloid in autoimmune diseases.

Inhibition of Treg Function: Concerns for Establishment of Pregnancy?

For the successful establishment of pregnancy, the mother’s immune system must be able to tolerate the presence of any paternally derived antigens that the fetus may express. Several mechanisms are in place to allow for the development of immunologic tolerance to allo-fetal antigens (Aluvlihare et al., 2004). For example, to prevent maternal rejection of the fetus, fetal tissue causes the breakdown of tryptophan, which results in inhibition of lymphocyte proliferation. Additionally, trophoblasts express human leukocyte antigen-G (HLA-G) and Fas ligand, which leads to the inhibition of NK cell activity and may induce apoptosis of activated Fas-expressing maternal lymphocytes, respectively. In the past 6 years, there has been accumulating evidence that maternal Tregs may play a role in maternal tolerance of the fetus, especially during the establishment of pregnancy (Guerin et al., 2009) (Saito et al., 2007). By studying both syngeneic and allogeneic crossing of mouse strains, Aluvlihare et al. (2004) were able to demonstrate that the establishment of pregnancy in mice results in an increased proportion of CD4⁺CD25⁺ T cells with suppressive function in blood and other lymphoid organs early during embryogenesis and, furthermore, that depletion of these cells results in a decreased number of viable fetuses in allogeneic, but not syngeneic, mouse strain matings. In addition, the mating of CBA/J females to DBA/2J males (C × D mating) results in a high spontaneous abortion, whereas the mating of CBA/J females to BALB/c males (C × B mating) results in successful pregnancies. The C × B mating results in an increased number of IL-10-producing decidual CD25⁺CD4⁺ T cells early during pregnancy compared with the C × D matings. In addition, the adoptive transfer of Tregs from C × B–mated pregnant females can reduce the spontaneous abortion rate in C × D–mated pregnant females if the transfer occurs shortly after conception (Zenclussen et al., 2005). In human pregnancy, an increased proportion of circulating Tregs and the presence of Tregs in the decidua have been documented (Somerset et al., 2004) (Sasaki et al., 2004). Moreover, there is a correlation between a lack of Treg numbers and/or function in women suffering from preeclampsia or recurrent abortion (Guerin et al., 2009). These data make clear that inhibition of Treg function for cancer therapy may result in decreased fertility in female patients.

Treg FUNCTION AS A SAFETY ASSESSMENT CONCERN

As this review of the literature makes clear, assessing the risks of manipulation of Treg function is difficult because our understanding of the mechanisms behind natural immune suppression is still poor. It will be difficult to assess the safety of the manipulation of Treg function without knowing the relationship of Treg numbers/function and the establishment of immune tolerance and suppression of inflammation in both normal and diseased animals and humans. Currently, both nonclinical and clinical studies monitor circulating Tregs in the blood as a measure of Treg status and attempt to correlate those numbers with observed pathology. However, there is some question as to whether the measurement of circulating Tregs is an accurate reflection of the Treg population, as the majority of Tregs reside within tissues rather than in blood (Seddiki and Kelleher, 2008). Although studies of FoxP3-deficient mice and humans indicate the potential dangers of complete abrogation of Treg function from conception, it is not clear if depletion of a portion, or even the majority, of Tregs in an adult will result in similar inflammatory effects. As other types of immune cells with suppressor function have been documented both in vitro and in vivo, CD4⁺CD25⁺FoxP3⁺ Tregs likely represent a fraction of regulatory immune cells and the monitoring of this population may therefore underrepresent the overall self-suppressive capability of the immune system (Bach, 2003) (Joosten and Ottenhoff, 2008).

Nonetheless, data generated from CTX- and CNI-treated patients and from CCR4 antagonist molecules in nonclinical vaccination studies suggest that decreasing Treg numbers or function can lead to an increase in effector immune cell function (see Table 1). Therefore, the major safety risk involved with decreasing Treg numbers/function is the development of inflammatory responses. In nonclinical safety studies and clinical trials where decreasing Treg function is
the therapeutic goal, it will be important to monitor animals and patients for signs of inflammation, including increased circulating T-cell counts, signs of increased T-cell numbers in immune organs, production of inflammation-associated serum proteins, inflammatory serum cytokines, and histopathologic evidence of inflammation in organs that are exposed to bacterial antigens (such as the gastrointestinal tract and lung). In addition, there may be a fertility concern as data suggest that the presence of Tregs is important for the establishment of pregnancy. Therefore, depending on the indication and the target patient population, the impact of decreased Treg function on female fertility may be warranted prior to first-in-human dosing. What remains unclear is how steep the curve is between Treg function and undesired activation of the immune system: At what point does inhibition of Treg function significantly increase the risk of developing autoimmune or fatal inflammatory response to infection? There may be little risk of inhibiting Treg function in the relatively short-term treatment but a larger risk in the face of long-term treatment.

Increasing Treg numbers and/or function as seen with rapamycin, corticosteroids, statins, and low levels of arsenic has the potential for the diminution of chronic inflammatory disease and perhaps even preeclampsia and recurrent spontaneous abortion. Yet this same enhanced Treg function is also associated with a risk of decreased host resistance to infection. This is a common risk for all immunosuppressive mechanisms, yet difficult to predict in nonclinical safety studies. Monitoring patients for signs of infectious disease will be important when assessing the safety of a therapeutic candidate that directly or indirectly causes enhancement of Treg function. Another commonly perceived risk for chronic immunosuppressive therapies is the potential for an increased susceptibility to the development of cancer. As Treg function correlates with a decreased capacity to reject tumor cells in animal models, it may be possible that long-term treatment of patients with a Treg-enhancing therapeutic may render their immune system less able to eliminate cancerous cells. Further research is required to determine if this hypothetical risk is real.

Lastly, it is important to realize that species-specific differences in Treg function/activation may confound our ability to determine the full extent of the changes in the immune response if Tregs are a target for therapeutic intervention, as was seen with TGN1412. Therefore, safety assessment of therapeutics that target the Treg population will require carefully designed nonclinical and clinical studies, which can optimize the detection of any negative aspects to manipulation of Treg function.

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