

Regulatory T Cells: Differentiation and Function

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Abstract

The immune system of vertebrate animals has evolved to mount an effective defense against a diverse set of pathogens while minimizing transient or lasting impairment in tissue function that could result from the inflammation caused by immune responses to infectious agents. In addition, misguided immune responses to "self" and dietary antigens, as well as to commensal microorganisms, can lead

to a variety of inflammatory disorders, including autoimmunity, metabolic syndrome, allergies, and cancer. Regulatory T cells expressing the X chromosome-linked transcription factor Foxp3 suppress inflammatory responses in diverse biological settings and serve as a vital mechanism of negative regulation of immune-mediated inflammation. *Cancer Immunol Res*; 4(9): 721–5. ©2016 AACR.

Introduction

The immune system has evolved sophisticated mechanisms to detect and eradicate or neutralize pathogenic microorganisms. The benefits of these mechanisms are constrained, however, by the potential impairment or loss of organ function that could result from excessive inflammation or from the tissue damage associated with misguided immune response directed against self, dietary, or environmental antigens. Numerous protective mechanisms have evolved to limit the negative consequences of an emerging adaptive immune response, particularly to protect vertebrates against reactivity to self-antigens and to limit immune-mediated inflammation. Central to the maintenance of immune homeostasis is a distinct form of immunologic tolerance mediated by a subset of CD4⁺ T cells with potent immunosuppressive properties known as regulatory T cells (Treg). These cells are defined by stable expression of the lineage-specific transcription factor Foxp3 and high amounts of the interleukin (IL2) receptor α -chain (CD25). Treg-mediated suppression is a vital mechanism of negative regulation of immune-mediated inflammation and features prominently in autoimmune and autoinflammatory disorders, allergy, acute and chronic infections, and cancer (Fig. 1). Tregs have also been found to play a notable role in metabolic inflammation and tissue repair. The association of Tregs with a multitude of human diseases has prompted investigations into the therapeutic manipulation of Tregs. The emerging modalities of Treg-based therapies are context specific, augmenting their numbers and functional activity in inflammatory, allergic, and autoimmune diseases,

while inhibiting their suppressive mechanisms or depleting them in cancer.

Historic Perspective

More than half a century ago, Jacques Miller discovered the function of the thymus as the site of generation of a major class of lymphocytes—T cells—and observed that thymectomy before day 3 of life unexpectedly results in a wasting disease (1). Subsequent studies showed the thymus to be critical in establishing and maintaining immunologic self-tolerance. Nishizuka and Sakakura reported that neonatal thymectomy of normal mice between days 2 and 4 after birth resulted in the autoimmune destruction of the ovaries (2). The alleviation of neonatal thymectomy-induced autoimmunity by the adoptive transfer of thymocytes or splenocytes from adult mice led to the realization that a population of cells generated in the mouse thymus after 3 days of life mediates tolerance in a dominant, cell-extrinsic manner. In 1995, Sakaguchi and colleagues made a fundamental discovery of a subset of CD4⁺ T lymphocytes expressing high amounts of CD25 and capable of suppressing disease in 3-day-old thymectomized mice and in other experimental models of autoimmunity (3). In contrast, when CD25⁺ cells were removed from the population of naïve CD4 T cells adoptively transferred into T-cell-deficient recipients, autoimmune inflammation ensued. Isolated CD25⁺CD4⁺ T cells are unable to proliferate in response to T-cell receptor (TCR) stimulation *in vitro*, but instead suppress proliferative responses of naïve CD4⁺ T cells. This *in vitro* suppression assay is widely used to characterize suppressor activity of Tregs. However, this test does not fully reflect the *in vivo* suppressor function of Tregs. Indeed, numerous studies have provided examples of Tregs with a pronounced impairment in their *in vivo* suppressor activity being indistinguishable in their *in vitro* suppressor function (see a review of this area in ref. 4).

In addition to serving as a Treg cell marker, CD25 expression is essential for the differentiation and proliferative fitness of Tregs. Mice lacking the IL2R α or IL2R β chains suffer from massive systemic autoimmunity that can be reversed by the adoptive transfer of CD4⁺CD25⁺ T cells from wild-type mice (5, 6). IL2-deficient mice were also found to have a significantly reduced number of CD4⁺CD25⁺ cells. The lymphoproliferative disease seen in CD25-deficient mice was due to a defect in Treg-mediated

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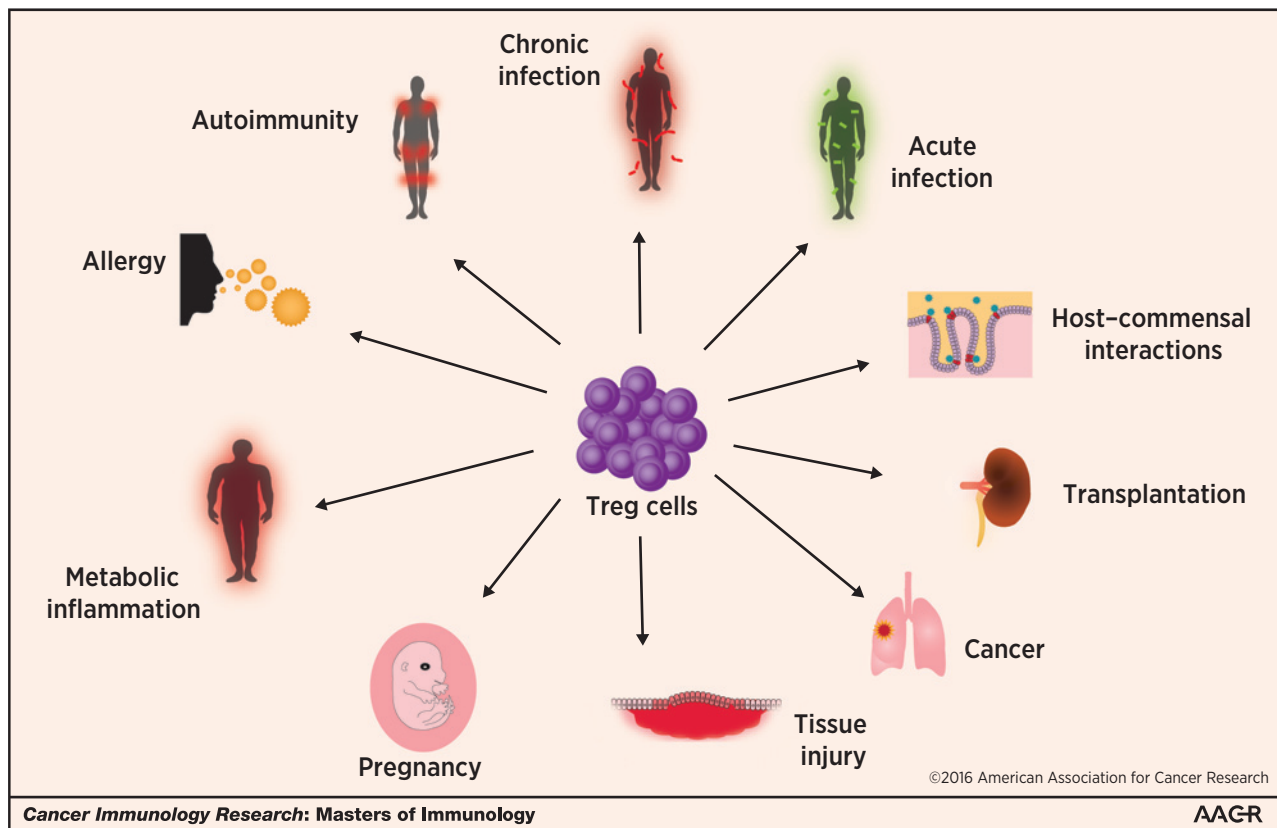


Figure 1.

Regulatory T cells function in multiple biological contexts, including autoimmunity, cancer, acute and chronic infections, host-commensal interactions and inflammation at barrier sites, allergy, pregnancy, tissue repair, metabolic sterile inflammation, and allo-transplantation.

suppression, because mice reconstituted with a mixture of wild-type and IL2R-deficient bone marrow did not exhibit autoimmunity (5, 6). Administration of an IL2-neutralizing antibody leads to a depletion of Tregs with consequent autoimmunity. Conversely, administration of an IL2-anti-IL2 complex, in which the antibody does not block the binding of IL2 to its receptor, results in massive Treg cell expansion. Thus, Tregs are dependent on IL2 signaling for their maintenance.

Foxp3 and Treg Cell Lineage Specification

The discovery of CD25 as a marker of Tregs raised a concern that Tregs may not represent a distinct T-cell lineage, but a particular state of activation, because CD25 is upregulated by all activated T cells. Other highly expressed markers on CD25⁺ Tregs, including CTLA-4 and GITR, are also upregulated upon activation of T cells. However, adoptive transfer of CD4⁺CD25⁺ T cells into lymphopenic mice showed that they proliferate in an MHC-dependent manner and, despite a decline in CD25 expression, their suppressive capacity increases (7). This finding and others spurred an intense exploration of potential genetic mechanisms underlying the differentiation and function of Tregs and a search for a more specific marker of these cells.

These efforts resulted in identification of Foxp3, an X chromosome-encoded member of the forkhead transcription factor

family, as a specific marker of Tregs (8–10). Foxp3 was identified as the gene whose loss-of-function mutation is responsible for a disease in mutant mice that spontaneously develop fatal, widespread, early-onset autoimmunity, and in humans with a rare inherited autoimmune disorder known as IPEX (immunodysregulation polyendocrinopathy, enteropathy X-linked syndrome; refs. 11–13). Foxp3 mRNA and protein are highly expressed in Tregs (8–10). Transduction of CD25⁻CD4⁺ T cells with Foxp3 retroviral expression vectors *in vitro*, or expression of a Foxp3 transgene in the majority of T cells *in vivo*, results in acquisition of a Treg cell phenotype, including suppressive activity, reduced production of IL2, and upregulation of Treg cell-associated markers, such as CD25, CTLA-4, and GITR (8–10). Mixed bone marrow transfer experiments showed that CD25⁺ Tregs originated only from Foxp3-sufficient, but not Foxp3-deficient, hematopoietic precursor cells (8). Foxp3 is also highly expressed in human CD25⁺CD4⁺ T cells with suppressor function. However, activated human conventional T cells also upregulate Foxp3, but only transiently and at a significantly lower level in comparison with the characteristically stable, high Foxp3 expression in Tregs (14). Similar to what was seen in mice, Foxp3 gene transfer to naïve human CD4⁺ T cells results in acquisition of suppressor functions (15). These studies have demonstrated that Foxp3 acts as a lineage specification factor essential for the differentiation of Tregs.

Subsequent studies have demonstrated that the lack of Tregs is responsible for all the observed manifestations of disease and pathology in *Foxp3*-deficient mice and, by extension, in humans (4, 8, 16). First, it was documented using mice expressing a green fluorescent protein (GFP) reporter under control of the endogenous *Foxp3* gene locus that *Foxp3* protein expression is restricted to a subset of CD4⁺T cells with suppressor function. Although the majority of *Foxp3*⁺T cells expressed high amounts of CD25, *Foxp3*⁺CD25⁻T cells were detectable in secondary lymphoid organs and nonlymphoid tissues. Both CD25⁺ and CD25⁻*Foxp3*⁺CD4⁺T cells had largely similar transcriptional signatures and potent suppressor function (16). Mice in which a conditional *Foxp3* allele in the T-cell lineage or in the germline were phenotypically indistinguishable—both types of mutant mice had fatal T cell–dependent autoimmune disease with identical onset, progression, and severity, indicating that expression of *Foxp3* in T cells is essential for control of the disease (16). Ablation of a conditional allele of *Foxp3* in thymic epithelial cells, macrophages, mammary gland epithelium, or dendritic cells (DC) did not have any detectable functional consequences (for review see ref. 4). Accordingly, transfer of Tregs into mice with the germline *Foxp3* deficiency rescued them from disease. Additional experiments have shown that *Foxp3* was dispensable for cell-intrinsic mechanisms of thymic and peripheral tolerance. Deletion of self antigen–specific thymocytes ("negative selection") was not dependent on *Foxp3*, nor was the activation and clonal expansion of peripheral antigen-specific T cells, nor was their production of cytokines (8, 16). Collectively, this series of experiments provided definitive proof that all clinical manifestations of *Foxp3* deficiency are due to the lack of functional Tregs.

Maintenance of Treg Cell Lineage

Foxp3 is not only required for differentiation of Tregs; it is also required for their suppressor function. This notion is supported by the observation that only CD4⁺T cells expressing a functional *Foxp3* GFP reporter allele (Treg "wannabe's"), but not those expressing a *Foxp3* GFP reporter null allele, are suppressive. *Foxp3* protein expression also confers fitness to Tregs (17). Loss of *Foxp3* expression by fully differentiated Tregs results in a loss of suppressor function and cell identity (18). This finding has raised concerns that differentiated Tregs may readily lose *Foxp3* expression in steady-state or inflammatory conditions. However, inducible genetic cell-fate mapping showed the remarkable stability of differentiated Tregs and that maintenance of *Foxp3* expression was heritable over the lifespan of animals under physiologic and inflammatory conditions (19). Stable *Foxp3* expression is acquired after a period of instability in newly generated Tregs (20). These studies suggest that Tregs represent a dedicated lineage. A dedicated mechanism for Tregs has been highlighted by demonstration that another *Foxp3* cis-regulatory element, CNS2, is essential for heritable maintenance of Treg cell identity during cell division when proinflammatory cytokines, promoting alternative cell fates, are present (21, 22). Thus, CNS2 enforces stability of the differentiated cell state in the Treg cell lineage in fluctuating environments, and this unique function of CNS2 is important in diverse types of chronic inflammation, including autoimmunity, chronic infection, metabolic inflammation, and cancer (22).

Thymic and Extrathymic Differentiation of Tregs

The identification of *Foxp3* as a specific marker for Tregs also shed considerable light on Treg cell differentiation. Based on the neonatal thymectomy studies, it had become abundantly clear that the thymus is an essential source for Tregs. The differentiation of thymic-derived Tregs (tTreg) depends on high-avidity interactions with self-peptide/MHC class II complexes and IL2 receptor signaling (4, 23). It became apparent, however, that Tregs could also be generated outside the thymus. These extrathymically generated or peripheral Tregs (pTreg) develop from naïve T-cell precursors upon exposure to antigenic stimulation under tolerogenic conditions. Specifically, strong TCR signaling, suboptimal costimulation, and high amounts of TGFβ and retinoic acid favor the induction of *Foxp3* in peripheral naïve CD4⁺T cells (4, 24). Evidence that the biological functions of tTregs and pTregs may be distinct came from the finding that an intronic *Foxp3* cis-regulatory element, CNS1 (conserved noncoding sequence 1), which contains Smad3- and retinoic acid receptor (RAR)-binding sites, is required for efficient differentiation of pTregs, but is dispensable for tTreg cell differentiation (21, 25). The bulk of CNS1 is encoded by a retrotransposon, which is found only in placental mammals. This observation suggested that the extrathymic differentiation of Tregs may have emerged during evolution of placental mammals to enforce maternal–fetal tolerance. Indeed, allogeneic pregnancy in CNS1-deficient mice results in increased embryo loss and defective spiral artery remodeling resembling pre-eclampsia in humans (25). A selective defect in the generation of pTregs leads to a late-onset allergic and asthma-like inflammation in the gut and lung (20). The gut is the main site of extrathymic differentiation of Tregs, where short-chain fatty acids, in particular butyrate produced by commensal microorganisms during starch fermentation, facilitates extrathymic generation of Tregs (for review see ref. 26). Collectively, the differences between these Treg cell subsets suggest that tTregs are likely responsible for tolerance to self-antigens, whereas pTregs restrain immune responses to "non-self," including allergens, commensal microbiota, and dietary antigens at barrier sites and paternal alloantigens. Relative contribution of tTregs and pTregs to progression to different cancer types remains to be established.

Suppressor Function of Tregs and Their Therapeutic Manipulation

The suppressor function of Tregs is essential for the restraint of fatal autoimmune and inflammatory responses throughout the lifespan of an organism. This has been demonstrated through the generation of knock-in mice expressing diphtheria toxin receptor (DTR) under control of the *Foxp3* locus. Elimination of Tregs in adult healthy *Foxp3*^{DTR} mice upon repeated administration of diphtheria toxin (DT) results in a disease similar to that found in *Foxp3*^{null} mice and death within 10 to 14 days with massive expansion and activation of granulocytes, DCs, NK cells, CD4⁺ and CD8⁺T cells, and B cells (27). CD4⁺T cells with a specificity for foreign antigens do not undergo activation upon Treg cell depletion, suggesting that T-cell activation in Treg cell–ablated mice is limited to T cells specific for "self."

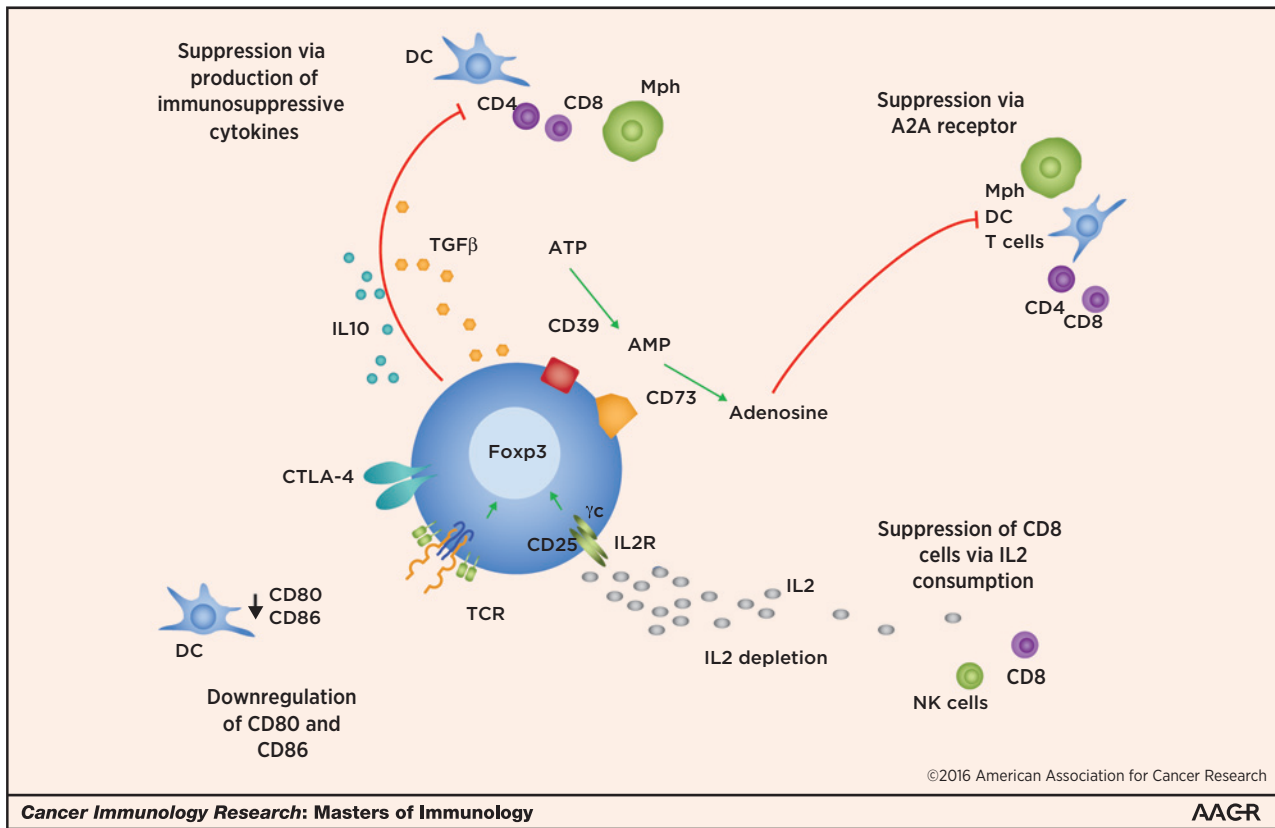


Figure 2.

Tregs control inflammation and all known types of the immune response by using diverse mechanisms of suppression including, but not limited to (i) production of immunomodulatory cytokines, such as IL10 and TGFβ, capable of suppressing proinflammatory responses of T cells, NK cells, B cells, DCs, and macrophages (Mph); (ii) enzymatic activity of CD39 and CD73 ectoenzymes highly expressed on the Treg cell surface, which enables conversion of extracellular ATP (serving as a proinflammatory mediator) to AMP and to adenosine, which then acts through adenosine receptors expressed by innate and adaptive immune cells (T cells, B cells, Mph, and DCs) and other cell types to suppress inflammatory responses; (iii) consumption of IL2 by Tregs, due to high expression of CD25, the high-affinity subunit of the IL2 receptor, restrains responses of CD8 T cells and likely NK cells; and (iv) high expression of CTLA-4 enables downregulation of costimulatory molecules CD80 and CD86 expression on DCs (4, 28). Suppressor function of Tregs is potentiated by signals through TCR and IL2 receptor (30, 31).

Transcriptional profiling of Tregs revealed a substantial number of candidate genes with the potential to mediate suppression (Fig. 2), including the highly expressed CD39 and CD73 ectoenzymes. These enzymes facilitate conversion of extracellular ATP to adenosine, which directly inhibits the proliferation of effector T cells and suppresses DCs and myeloid cells. Tregs also produce a number of secreted immunomodulatory proteins (e.g., IL10, granzymes, and TGFβ). IL10 production by Tregs is essential for keeping the immune response in check at environmental interfaces such as the colon and lungs (for review see ref. 4). High CTLA-4 expression by Tregs may enable them to suppress CD80 and CD86 expression by DCs (28). Interestingly, although congenital deficiency of CTLA-4 specifically in Tregs is associated with a lethal lymphoproliferative disorder, conditional ablation of CTLA-4 in differentiated Tregs in adult mice does not precipitate systemic autoimmunity, suggesting the existence of compensatory regulatory mechanisms independent of CTLA-4 (29). Finally, a recent study showed that IL2 consumption by Tregs is essential for control of CD8 T-cell responses, but is dispensable for control of CD4 T-cell acti-

vation and expansion (30). Although Tregs use multiple mechanisms of suppression and no single mechanism can account for the entire spectrum of their suppressor function, TCR and IL2 receptor signaling are required for the suppressor activity of Tregs (30, 31).

It has been established that Tregs play a role in spontaneous autoimmune and inflammatory responses and have proven capacity to exert potent suppressive effects in preclinical models of diverse immune-mediated pathologies, including autoimmune diabetes, inflammatory bowel disease, multiple sclerosis, allogeneic transplantation, and allergy. Thus, numerous strategies to manipulate these cells for therapeutic benefits are being developed. The first successful test was of graft-versus-host disease in patients undergoing allogeneic bone marrow transplantation for the treatment of hematologic malignancies. The therapy was based on adoptive transfers of *ex vivo*-expanded Tregs (32, 33).

Ongoing work is multi-pronged, involving the development of adoptive Treg cell therapies, biologics, microbial cocktails, and small molecule-based approaches to boost Treg cell numbers for treatment of autoimmune and inflammatory diseases

and prevent transplant rejection. Increased numbers of Tregs have been observed in different solid organ cancers in humans, where it has been associated with poor prognosis. Consistent with these observations, Treg cell depletion has a pronounced therapeutic effect in animal cancer models (34). These preclinical and clinical findings form the basis for the current efforts to develop effective methods for transient generalized depletion of Tregs, local depletion, or inactivation of intratumoral Tregs for cancer therapy. Thus, the advances in our understanding of the basic principles of Treg cell biology are beginning to translate into clinical practice.

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