

FOXP3: Of Mice and Men

Steven F. Ziegler

Immunology Program, Benaroya Research Institute and Department of Immunology, University of Washington School of Medicine, Seattle, Washington 98101; email: sziegler@vmresearch.org

Annu. Rev. Immunol.
2006. 24:209–26

First published online as a
Review in Advance on
December 1, 2005

The *Annual Review of
Immunology* is online at
immunol.annualreviews.org

This article's doi:
10.1146/annurev.immunol.24.021605.090547

Copyright © 2006 by
Annual Reviews. All rights
reserved

0732-0582/06/0423-0209\$20.00

Key Words

regulatory T cell, autoimmunity, forkhead, transcription factor

Abstract

The immune system has evolved mechanisms to recognize and eliminate threats, as well as to protect against self-destruction. Tolerance to self-antigens is generated through two fundamental mechanisms: (a) elimination of self-reactive cells in the thymus during selection and (b) generation of a variety of peripheral regulatory cells to control self-reactive cells that escape the thymus. It is becoming increasingly apparent that a population of thymically derived CD4⁺ regulatory T cells, exemplified by the expression of the IL-2R α chain, is essential for the maintenance of peripheral tolerance. Recent work has shown that the *forkhead* family transcription factor Foxp3 is critically important for the development and function of the regulatory T cells. Lack of Foxp3 leads to development of fatal autoimmune lymphoproliferative disease; furthermore, ectopic Foxp3 expression can phenotypically convert effector T cells to regulatory T cells. This review focuses on Foxp3 expression and function and highlights differences between humans and mice regarding Foxp3 regulation.

Foxp3: forkhead box protein P3

Tregs: regulatory T cells

CD4⁺CD25⁺

Treg: a subset of CD4⁺ T cells that is capable of suppressing the proliferation and cytokine production of naive or memory T cells

Forkhead family: a large family of transcriptional regulators named after the founding member, which was found to be the gene responsible for the *forkhead* mutation in *Drosophila*. All members of the family have a closely conserved motif, known as a forkhead box, or Fox, that is involved in DNA binding. The family is further subdivided based on homology outside the forkhead box.

INTRODUCTION

Immunological tolerance to self-antigens is a tightly regulated process. A primary mechanism for self-tolerance is deletion of self-reactive cells in the thymus. However, this mechanism is not perfect, and autoreactive clones do escape into the periphery. Tolerance is maintained in the periphery through a variety of mechanisms, including a population of regulatory T cells that actively suppress the function of autoreactive T cells. These T cells, identified by their expression of CD4, the IL-2R α chain (CD25), and the *forkhead* family transcription factor Foxp3, are known as regulatory T cells, or Tregs. They have the ability to inhibit the development of autoimmunity when transferred into the appropriate host. Recent work has shown that the *forkhead*/winged-helix protein Foxp3 is expressed predominantly in Tregs and is both necessary and sufficient for their development and function.

Several excellent reviews have been written recently on the development and function of CD4⁺CD25⁺ Tregs (1–5). Thus, this review does not directly address these issues. Instead, I review what is known concerning the expression and function of Foxp3, which is critical for the development and function of Tregs, especially in the mouse. Although the overall importance of Foxp3 is obvious from the phenotype of humans and mice that lack this protein, very little is actually known about how it functions and what controls its expression. This review highlights the similarities and differences in Foxp3 expression function in human and mouse, with an emphasis on the differences.

IDENTIFICATION AND CHARACTERIZATION OF MOUSE Foxp3

Characterization of *Scurfy* (*sf*) Mutant Mice

The *scurfy* (*sf*) mutation arose spontaneously in the partially inbred MR strain in 1949 at the

Oak Ridge National Laboratory. Subsequent studies showed the gene to be X-linked, with only X^{*sf*}/Y males affected (here, these mice are referred to as *sf*/Y). Grossly, affected *sf*/Y males exhibit several external markers of disease, including ear thickening; scaling of the ears, eyelids, feet, and tail; and severe runting. Internally, the mice exhibit lymphadenopathy, splenomegaly, hepatomegaly, and massive lymphocytic infiltrates in the skin and liver. These lesions closely resemble those seen in a graft-versus-host reaction. Mice display anemia and have a positive direct Coombs's test, but they lack evidence of autoantibodies against double-strand DNA or small ribonuclear protein antigens (6). Taken together, these gross assessments suggest that *sf* is a mutation that causes an autoimmune-like disease in affected animals. In this regard, *sf*/Y mice resemble mice bearing targeted mutations in the *ctla-4* or *tgf- β 1* genes. These mice die at approximately three weeks of age from a massive lymphoproliferative disease, with peripheral lymphocyte levels up to 20-fold greater than normal mice (7, 8).

Phenotypically, *scurfy* disease is most consistent with a diagnosis of autoimmune lymphoproliferative disease. This idea is supported by the finding that mice expressing a transgenic T cell receptor (TCR) survive significantly longer in the absence of antigen stimulation (60 days compared with 20–24 days) and that they live a normal lifespan and are free of *sf* disease when they are also *rag-2*-null (*sf*/*ova*/*rag* mice). Finally, T cells from *sf*/Y mice display reduced sensitivity to inhibitors of T cell activation, suggesting that TCR signaling is dysregulated in *sf*/Y mice. Taken together, these data suggest the *sf* disease results from an inability to properly regulate antigen-driven T cell activation.

One of the *in vitro* hallmarks of *sf* disease is the spontaneous proliferation and cytokine production exhibited by T cells isolated from *sf*/Y mice. Initial studies showed that cytokine production *in vitro* by ConA-stimulated *sf*/Y splenocytes was greatly elevated (9). More detailed studies, using purified

CD4⁺ T cells from *sf/Y* and normal littermate control (NLC) mice, showed that these cells were capable of proliferation and cytokine production when examined directly *ex vivo*. However, to achieve maximal T cell stimulation, TCR engagement was required, suggesting that the defect in *sf* mice is not nonspecific T cell activation. CD4⁺ T cells from *sf/Y* mice were hyperresponsive to TCR-mediated signals, responding to TCR engagement with crosslinking antibodies at concentrations 10-fold lower than their wild-type counterparts (10). Consistent with the increased expression of costimulatory molecules in *sf/Y* mice, CD4⁺ T cells from these mice responded to CD28 crosslinking (10). Recent studies have shown that the transcription factors NFAT and AP-1 are constitutively activated in CD4⁺ T cells from *sf/Y* mice (11). Also, these same T cells have a marked decrease in their sensitivity to cyclosporin A, with an IC₅₀ 15-fold higher than NLC-derived T cells (10). Both observations are indicative of an alteration in TCR-mediated signal transduction in CD4⁺ T cells from *sf/Y* mice and may account for the increased cytokine production exhibited by these cells.

Consequence of Foxp3 Expression on CD4⁺ T Cell Function

The gene responsible for *scurfy* disease was molecularly cloned using a standard positional cloning approach (12). Sequence analysis of the cloned gene showed that it encoded a novel member of the *forkhead* family of transcriptional regulators, Foxp3 (see below for more details). To verify this gene as the gene responsible for the *scurfy* mutation, transgenic mice were generated using a cosmid clone containing mouse *Foxp3*. When bred to *sf/Y* mice, expression of the transgene was capable of rescuing the mice from *scurfy* disease and demonstrated that a mutation in *Foxp3* was indeed responsible for the *scurfy* phenotype (12).

The Foxp3 transgenic mice (referred to as Foxp3-Tg hereafter) also provided a model

system for examining the *in vivo* consequences of Foxp3 expression. Several individual Foxp3-Tg lines were established, each with differing levels of transgene expression (12, 13). Each of these lines, when bred into an otherwise wild-type background, resulted in a reduction in peripheral CD4⁺ and CD8⁺ T cell numbers, with the extent of the reduction directly correlated with transgene copy number and expression (12). However, thymic cellularity was unaffected, as was positive and negative selection. Thus, levels of Foxp3 determined the number of peripheral T cells, while having little effect on the number and differentiation of thymocytes.

To examine the function of T cells from Foxp3-Tg mice, a single transgenic line was chosen. This line (2826) had approximately 16 copies of the transgene and had approximately fivefold higher levels of Foxp3 expression (13, 13a). Although T cell development appears to be normal in these mice, peripheral T cell numbers and representation in secondary lymphoid organs are dramatically decreased. Using a variety of *in vitro* and *in vivo* assays, the function of T cells from the Foxp3-Tg mice was shown to have severely decreased responses when activated through the TCR. For example, purified CD4⁺ T cells displayed dramatically reduced proliferative responses when stimulated *in vitro* (13). Similarly, these T cells produced virtually no IL-2 upon stimulation. In fact, by any measure, CD4⁺ T cells from Foxp3-Tg mice were functionally inert. Taken together, these data reflect a generalized deficit in cellular activation of CD4⁺ T cells that express Foxp3.

The failure of T cells from Foxp3-Tg mice to function *in vitro* is also reflected in the response of these mice to immunologic challenge *in vivo*. The Foxp3-Tg mice failed to mount an antigen-mediated contact sensitivity response (13). Also, recent data demonstrate that Foxp3-Tg mice have dramatically depressed responses to T-dependent antigens (14). The latter response appears to be due to a reduced ability to produce cytokines and to

NFAT: nuclear factor of activated T cells

elaborate CD40L on their cell surface. Thus, overexpression of Foxp3 in CD4⁺ T cells *in vivo* results in lowered overall numbers of T cells, with impaired functionality of those T cells that remain.

Foxp3: Necessary and Sufficient for Mouse CD4⁺CD25⁺ Treg Development and Function

Although it is clear that expression of Foxp3 in CD4⁺ T cells has deleterious effects on their ability to respond normally to TCR-mediated signals (13, 14), the fact that a population of Foxp3⁺ T cells exists suggests a role for this protein in CD4⁺ T cell function. Clues for this function come from an initial analysis of the cells that express Foxp3 in the mouse. Several groups simultaneously reported that the predominant cell type that expressed Foxp3 was CD4⁺CD25⁺ T cells (15–18), the population of CD4⁺ T cells that can suppress the proliferation and cytokine production of TCR-stimulated conventional CD4⁺ T cells (19, 20). The role of Foxp3 in these cells has been elucidated in mice through a combination of genetic and direct functional studies. Taken as a whole, these data demonstrate that Foxp3 expression in mouse CD4⁺ T cells is sufficient to mark these T cells as regulatory. CD25, the IL-2R α chain, had previously been shown to be the only reliable marker for these cells (19); however, it is also a marker of activated CD4⁺ T cells. Thus, the identification of Foxp3 as a marker for this population of T cells was critical for the further analysis of these cells and their role in peripheral tolerance.

In addition to the correlation of Foxp3 expression and CD4⁺CD25⁺ Tregs, the ability of Foxp3 to reprogram CD4⁺ T cell function has recently been reported (15–17). Several lines of evidence demonstrate a role for Foxp3 in the development and/or function of CD4⁺CD25⁺ Tregs. First, as described earlier, CD4⁺CD25⁺ T cells display Foxp3 expression, whereas other T cell subsets in the mouse do not have detectable expression (15,

16). In fact, using a knockin allele of *Foxp3* consisting of an in-frame fusion of green fluorescent protein (GFP) and Foxp3, Fontenot et al. (21) showed that $\alpha\beta$ TCR⁺CD4⁺ T cells are the predominant cell population that expresses Foxp3. They also found that the relative number of CD25⁺ and CD25⁻ cells that were Foxp3⁺ varied according to the source of the T cells. The ability of the Foxp3⁺ T cells to act as suppressors *in vitro* was not dependent on expression of CD25. These data were confirmed using a second knockin mouse containing an IRES-RFP (internal ribosome entry site–red fluorescent protein) cassette in the 3' untranslated region of the Foxp3 gene (22).

The second line of evidence that Foxp3 expression is necessary and sufficient for mouse Treg development and function comes from studies using ectopic expression of Foxp3 in otherwise conventional T cells. Infection of CD4⁺CD25⁻ T cells with a retrovirus expressing Foxp3 converted those cells to a regulatory phenotype, with the infected cells capable of suppressing the proliferation of uninfected CD4⁺CD25⁻ T cells (16). The infected cells can also function as Tregs *in vivo*. In a wide variety of adoptive transfer models of autoimmune disease, cotransfer of CD4⁺CD25⁺ T cells has been shown to protect the host from disease development in these model systems (23–26). Using naive CD4⁺CD25⁻ T cells expressing Foxp3 following retroviral gene transfer, Hori et al. (16) showed that cotransfer of cells infected with the Foxp3 retrovirus also protects host mice from autoimmune gastritis. Similarly, CD4⁺CD25⁻ T cells expressing Foxp3 also protects against colitis (15). These results have been confirmed by several groups, demonstrating that expression of Foxp3 can convert cells to a Treg-like phenotype. Recent studies have suggested a potential therapeutic use of ectopic Foxp3 expression. BDC2.5 TCR transgenic T cells, transduced with a Foxp3-expressing retrovirus, were capable of ameliorating disease when transferred into nonobese diabetic (NOD) mice with recent

onset disease (27). However, transfer of polyclonal NOD CD4⁺ T cells infected with the Foxp3 retrovirus did not protect, suggesting that the frequency of antigen-specific Tregs is a major factor in the ability of Tregs to control autoimmunity. Similar results have been seen with transfer of purified CD4⁺CD25⁺ Tregs (25, 28).

Complimentary to the experiments using retroviral-mediated delivery of *Foxp3* to T cells, Treg function in Foxp3-Tg animals has also been studied. As described above, these mice contain a transgene consisting of cosmid clone containing the mouse Foxp3 gene (12), with approximately 80% of CD4⁺ T cells in these animals expressing Foxp3 (D.J. Kaspirowicz & S.F. Ziegler, unpublished observation). An examination of the function of the CD4⁺ T cells from these mice showed that the entire population had in vitro regulatory activity (13a, 17). Thus, similar to what was seen with retroviral-mediated introduction of Foxp3, transgenic expression also converted conventional CD4⁺ T cells to a regulatory phenotype. Interestingly, CD8⁺ in these mice also displayed in vitro regulatory activity, demonstrating that Foxp3 expression in non-CD4⁺ T cells was also capable of phenotypic conversion.

Just as ectopic Foxp3 expression has been shown to drive Treg function, lack of Foxp3 has been correlated with a lack of Treg cells. For example, mice that lack functional Foxp3, either via the *scurfy* mutation or a targeted mutation, lack Treg activity (15, 17, 21). Importantly, a conditional deletion of *Foxp3* in CD4⁺ T cells also led to a lymphoproliferative disease indistinguishable from that seen in *scurfy* males (21). Further evidence for the critical role of Foxp3 in determining the Treg lineage comes from mixed bone marrow chimera experiments. Lethally irradiated mice were reconstituted with a mixture of bone marrow from wild-type and *Foxp3*⁻ mice, congenically marked to allow the contribution of each to the reconstituted animal. Only bone marrow from the wild-type donor contributed to the CD4⁺CD25⁺ compartment, demonstrat-

ing that Foxp3 is needed in the development of Tregs and that the role of Foxp3 is cell intrinsic (15).

Taken as a whole, the data described in this section demonstrate the absolute need for Foxp3 in the development and function of Tregs. They also show that ectopic expression of Foxp3 can cause a phenotypic conversion of T cells to a regulatory phenotype. Thus, in the mouse, Foxp3 is both necessary and sufficient for Treg development and function.

Regulation of Foxp3 expression. Although obviously important to the understanding of Treg development, the factors that regulate *Foxp3* expression remain elusive. The primary issue that has confounded these studies is that the factors that appear to affect Foxp3 expression also affect the survival and expansion of Tregs. However, some signaling pathways, including CD28, IL-2, and TGF- β , are emerging that appear to have an effect on the expression of *Foxp3*. For example, recent work has shown that CD28-mediated signals during thymic development are required for proper *Foxp3* expression (29). However, other studies have implicated CD28 in Treg survival and expansion (30, 31), complicating an interpretation of the role of CD28 in *Foxp3* expression. Similarly, mice lacking the IL-2 pathway owing to targeted mutations also display a deficit in CD4⁺CD25⁺ Tregs. This is true for mice with mutations in *il-2*, *il-2r α* , and *il-2r β* , as well as for mice lacking the downstream mediator of IL-2 signaling, Stat5 (32–40). However, similar to the situation with CD28, distinguishing between a direct affect on transcription and survival is not possible. Recent studies have shown that IL-2 is absolutely required for Treg expansion in the periphery (41, 42). Furthermore, Malek et al. (39, 40) showed that thymus-restricted expression of IL-2R β -chain was sufficient to rescue *il-2rb*^{-/-} mice from fatal autoimmune disease, demonstrating a need for IL-2 signals during thymic development of Tregs. Finally, using the GFP-Foxp3 strain, Fontenot et al. (21) have shown that thymic expression of Foxp3

TGF- β :
transforming growth factor- β

Stat: signal transduction activator of transcription

is absolutely dependent on TCR-MHC interactions and independent of commitment to either the CD4 or CD8 lineage. A more definitive assessment of the factors that control *Foxp3* expression await an analysis of its *cis*-regulatory region. The availability of the knockin strains described above, as well as a characterization of the *Foxp3 cis*-regulatory region, will be invaluable in sorting out direct and indirect effects of these and other factors on *Foxp3* expression.

Perhaps the most controversial aspect of *Foxp3* gene regulation is the role of TGF- β . Although TGF- β can have effects on Treg expansion and survival (43, 44), its actual role is not at all clear. Several groups have shown that in vitro culture of CD4⁺CD25⁻ T cells with a cocktail that includes TGF- β and IL-2, in combination with TCR engagement and costimulation through CD28, can lead to induction of *Foxp3* expression and acquisition of repressor activity (45–47). However, there are several issues with these experiments. The cultures used superphysiological concentrations of TGF- β , and investigators have shown that TGF- β can inhibit the proliferative effects of IL-2, at least in conventional T cells (48). Thus, one explanation for the in vitro results is that TGF- β inhibits the proliferation of CD4⁺CD25⁻*Foxp3*⁻ T cells in the cultures, allowing the expansion of contaminating *Foxp3*⁺ cells and an apparent increase in *Foxp3* mRNA. Also, the culture systems used are very likely to produce a complex combination of signals in responding cells, making a definitive role for any of the signaling molecules difficult to discern. In support of an indirect role for TGF- β in *Foxp3* expression and function, Tregs from mice either lacking TGF- β 1 or expressing a dominant-negative TGF- β R2 are indistinguishable from their counterparts from wild-type mice in in vitro suppression assays (49).

The data on the in vivo role of TGF- β are equally contradictory. In support of a role for TGF- β in Treg function and *Foxp3* expression, several groups have shown that TGF- β is capable of expanding the in vivo

pool of Tregs (18). For example, T cell-specific expression of a dominant-active form of TGF- β leads to an elevation in the number of CD4⁺CD25⁺ Tregs and increased *Foxp3* expression (50, 51). However, these studies do not differentiate between expansion and de novo generation of Tregs. Using mice with an inducible TGF- β transgene, Peng et al. (44) showed that transient TGF- β expression specifically in the pancreatic islets was sufficient to protect against onset of diabetes. They found that this transient expression increased the number of CD4⁺ T cells with a regulatory phenotype in the islets, consistent with a role for TGF- β in the expansion of Tregs in vivo. Studies using mice incapable either of producing TGF- β or of responding to it provide the most contradictory data. These mice have been used to demonstrate that TGF- β is either absolutely required for Treg expansion and function (50, 51) or completely dispensable (49). In addition, recent work has suggested that the effector T cell needs to be TGF- β responsive in order to be suppressed, at least in vivo (52). However, this appears not to be the case in vitro (49; K. Newton & S.F. Ziegler, manuscript in preparation). The recent development of *Foxp3* reporter mice should help clarify this issue. In fact, using mice with a reporter for *Foxp3* mRNA, Wan & Flavell (22) showed that TGF- β may directly influence *Foxp3* expression.

FUNCTIONAL CHARACTERIZATION OF *Foxp3*

As described above, *Foxp3* is a member of the *forkhead*/winged-helix family of transcriptional regulators. A common feature of members of this family is the FKH domain (**Figure 1**), which has been shown to be necessary and sufficient for DNA binding (53, 54). DNA-binding analyses from a number of Fox family proteins have defined a core DNA sequence (5'-A(A/T)TRTT(G/T)R-3'', where R = pyrimidine) surrounded by less conserved sequences (55). Experimentally, two

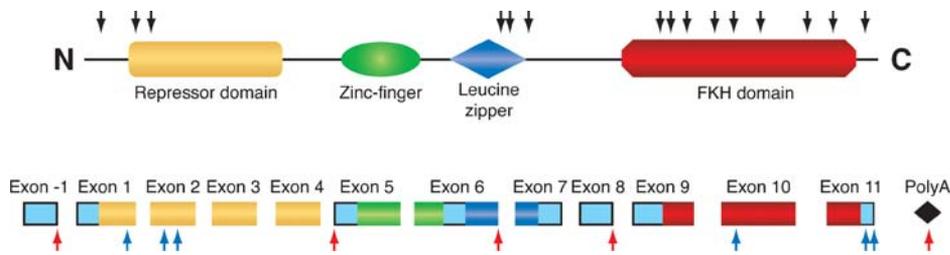


Figure 1

Schematic of human FOXP3 and *FOXP3* with location of known IPEX mutations. Top schematic is FOXP3, with locations of missense mutations indicated by arrow. Bottom schematic is *FOXP3*, with location of mutations predicted to affect splicing or mRNA stability (*red arrows*) and to generated frameshifts (*blue arrows*) indicated. Exons are color coded according to the protein schematic, with pale blue indicating coding region of unknown function (FKH: forkhead).

canonical FKH-binding sequences [from the transthyretin promoter (TTR-S) and the immunoglobulin variable regions V1 promoter (V1P) (56)] have been used as templates to analyze the DNA-binding properties of Fox proteins. Similar to other members of the Fox family, Foxp3 can bind to each of these FKH-binding sites (57). Thus, as predicted from the sequence analysis, Foxp3 is a DNA-binding protein capable of specific binding to a canonical FKH DNA-binding site.

Members of the Fox family are both transcriptional activators and transcriptional repressors. To assess the role of Foxp3 in transcriptional regulation, a reporter plasmid was constructed that contained three copies of the V1P FKH site linked to a minimal SV40 promoter and the firefly luciferase gene. Cotransfection of this reporter plasmid with a Foxp3 expression plasmid into Cos-7 cells resulted in a dramatic reduction of luciferase activity, suggesting that Foxp3 acts as a transcriptional repressor (57). The ability of Foxp3 to act as a transcriptional repressor required the presence of the FKH domain of Foxp3 and the V1P-binding site, showing that binding of Foxp3 to the FKH site is responsible for the observed effect of Foxp3 on the expression of the reporter.

Several lines of evidence suggest that a major target of Foxp3-mediated transcriptional regulation is cytokine genes. For ex-

Other Forkhead Family Proteins and Immune Regulation

In addition to Foxp3, several other members of the *forkhead* family have been implicated in regulating immune system development and function (90). For example, mutations in the *Foxn1* gene are responsible for the phenotype seen in nude (*nu*) mice, rats, and humans (91, 92). These mice are characterized by abnormal development of the epidermis, lack of hair, and absence of a thymus. Mutations in *Foxj1* lead to embryonic lethality, but fetal liver chimeras have been used to study its role in the immune system. These mice displayed multisystemic cellular autoimmunity, as well as T cell hyperproliferation and hyperactivity. CD4⁺ T cells from these animals were skewed toward a Th1 cytokine profile, could be activated solely by IL-2 (CD3 and CD28 stimulation were not required), and exhibited decreased levels of the IκKβ regulatory subunit (93). These results are consistent with a role for Foxj1 in the negative regulation of NF-κB activity. Finally, Foxo3a has been implicated in cellular survival, where it is involved in the regulation of NF-κB (94, 95). Upon Akt-dependent phosphorylation, Foxo3a is shuttled to cytoplasm and rendered inactive as a transcriptional regulator. Mice deficient in Foxo3a develop T cell hyperactivity and multiorgan lymphocytic infiltrates with age.

ample, ectopic expression of Foxp3 in Jurkat cells resulted in a marked reduction of IL-2 production following stimulation (57). In addition, CD4⁺ T cells from mice expressing

a *Foxp3* transgene were unable to produce IL-2, IL-4, or IFN- γ following TCR-mediated stimulation in vitro and had severely reduced ability to express cytokines in vivo following immunization (13, 14). Taken as whole, these data demonstrate a role for Foxp3 in the negative regulation of cytokine gene expression in CD4⁺ T cells. Inspection of composite NFAT/AP-1 sites upstream of several cytokine genes revealed a possible explanation for the ability of Foxp3 to regulate these genes. Either overlapping or directly adjacent to these DNA-binding sites was a potential FKH-binding site. Provocatively, oligonucleotide probes containing the composite sites from the human IL-2 and GM-CSF genes were capable of competing with the VIP site for Foxp3 binding in a gel shift assay (57). Confirmation of the ability of Foxp3 to repress NFAT-mediated transcription comes from experiments using a reporter plasmid consisting of the -280 composite NFAT/AP-1 site from the mouse IL-2 gene. NFAT activation was inhibited when the Foxp3 expression vector was cotransfected (57). Furthermore, work from our laboratory, using a bipartite reporter containing both NFAT and GAL4 DNA-binding sites, has shown that Foxp3 can inhibit NFAT-mediated transcription while binding to a more distant DNA site (J. Lopes, T. Torgerson, L.A. Schubert, H. Ochs, and S.F. Ziegler, manuscript submitted). These experiments took advantage of fusion proteins consisting of the yeast GAL4 DNA-binding domain fused to the non-FKH sequences from FOXP3, which was found to be capable of inhibiting activation-induced expression of the NFAT reporter.

Much effort is currently being used to define functional domains within FOXP3. As shown in **Figure 1**, FOXP3 contains at least three distinct structural domains: FKH, Leucine zipper, and C2H2 zinc finger. As described above, the FKH domain is critical for both DNA binding and for nuclear localization. Confirmation of its role in nuclear localization comes from site-directed mutation of

two lysine residues (K415 and K416), at the carboxy end of the FKH domain, to glutamic acid. This mutant form of FOXP3, when expressed in cell lines, is localized to the cytoplasm (J. Lopes, T. Torgerson, L.A. Schubert, H. Ochs, and S.F. Ziegler, manuscript submitted).

The functional properties of the remainder of the protein are not well understood. However, studies on other members of the FOXP family may be relevant to the analysis of FOXP3 function. For example, the Leucine zipper domain of Foxp1 and Foxp2 is critical for homo- and heterodimer formation (58, 59). Additionally, deletion of the Leucine zipper domain from Foxp1 and Foxp2 abrogated their ability to act as transcriptional repressors. This domain of FOXP3 is likely also to be involved in dimerization (see below).

The amino terminal half of FOXP3 contains no obvious identifiable functional domains, although it does contain a fairly high proportion of proline residues (**Figure 1**). However, we have shown that a fusion protein containing the DNA-binding domain of the yeast transcription factor GAL4 and the amino terminal half of human FOXP3 (amino acids 1–198) was functional as a transcriptional repressor, whereas a fusion of the zinc finger and Leucine zipper domains was non-functional (the GAL4 DNA-binding domains also contain nuclear localization and dimerization sequences). Using infection of primary T cells with FOXP3 deletion mutants, Bettelli et al. (11) showed that there were two functional domains within the amino terminal 200 amino acids of Foxp3, one between amino acids 1 and 150, and one between amino acids 150 and 200. Our lab has also defined two functional domains with the amino terminus of Foxp3. One domain, between amino acids 67 and 132, is involved in general transcriptional repression by FOXP3. The second, between amino acids 135 and 198, is specifically required for repression of NFAT-mediated transcription. Bettelli et al. (11) also showed that Foxp3 can directly interact with NFAT

and the NF- κ B subunit P65. Although the region of interaction with Foxp3 was not identified, that data described above would predict that the region between amino acids 135 and 198 is involved.

HUMAN FOXP3: NOT QUITE THE SAME AS MOUSE

The identification of *Foxp3* as the gene mutated in *scurfy* mice led to an investigation of its possible role in a human syndrome with clinical features similar to those seen in *scurfy* animals. This syndrome, known as IPEX (immune dysfunction/polyendocrinopathy/enteropathy/X-linked), was first characterized in 1982 and subsequently mapped to the X chromosome (60, 61). It is characterized by watery, sometimes bloody, diarrhea in very early infancy. In addition, nearly all affected males develop dermatitis (usually eczematous in nature), and most cases develop autoimmune endocrinopathies, with type 1 diabetes and thyroiditis the most common. In general, affected individuals develop symptoms very early in infancy and usually die in the first two years of life (61–64).

The similarity of the phenotypes of *scurfy* mice and IPEX humans suggested a common cause for the two diseases. Indeed, mutations in *FOXP3* have been identified in over 20 families with affected offspring (62, 65–69). Many of these are missense mutations in the FKH domain, but mutations have been found across the length of the gene (Figure 1). However, not all patients diagnosed with IPEX have *FOXP3* mutations. Sequencing of the *FOXP3* gene in a large cohort of individuals diagnosed with IPEX revealed identifiable coding region mutations in 60%, with an additional 10% having dramatically reduced mRNA levels (65). One interpretation of these data is that a mutation in *FOXP3* leads to development of IPEX, and those IPEX patients without *FOXP3* mutations may be mutations in genes whose products interact with *FOXP3*, either physically or functionally. Indeed, a recent study has found a patient with IPEX-like

symptoms for whom *FOXP3* was ruled out as a candidate gene (66).

As shown in Figure 1, *FOXP3* mutations in IPEX patients have been identified throughout the coding region. We have begun to analyze the affect of these IPEX mutations on *FOXP3* function. Accordingly, mutations in the FKH domain ablate the ability of *FOXP3* to inhibit transcription of a reporter construct containing canonical forkhead-binding sites. Consistent with the prevailing view of the FKH domain, mutations in *FOXP3* that are predicted to affect DNA binding fail to inhibit transcription of a FKH-dependent reporter (J. Lopes, T. Torgerson, L. Schubert, H. Ochs, and S.F. Ziegler, manuscript submitted). Interestingly, two IPEX patients were shown to have single codon mutations in the Leucine zipper domain of *FOXP3*(Δ K250 and Δ E251) (70, 71). We have now found that introducing the Δ E251 mutation into *FOXP3* results in a failure to homodimerize and in reduced ability to repress transcription (J. Lopes, T. Torgerson, L.A. Schubert, H. Ochs, and S.F. Ziegler, manuscript submitted). These data strongly suggest that the Leucine zipper domain of *FOXP3* is critical for proper function. Additional mutations have been identified in the amino terminal domain identified as critical for *FOXP3* function (see previous section); these mutations are now being analyzed to determine their impact on *FOXP3* transcriptional repression.

Human FOXP3: Regulation of Expression and Multiple Isoforms

A population of CD4⁺CD25⁺ T cells has also been identified and characterized from human peripheral blood. These cells, when assayed in vitro, are anergic, have the ability to suppress the proliferation and cytokine production of cocultured CD4⁺CD25⁻ T cells, and express *FOXP3* (18, 72–75). Thus, as has been demonstrated in the mouse, human CD4⁺CD25⁺ T cells express *FOXP3* and act as suppressors.

IPEX: immune dysfunction/polyendocrinopathy/enteropathy/X-linked

However, further examination of FOXP3 expression showed several differences between humans and mice. When FOXP3 expression was monitored by Western blotting, it became apparent that the protein actually ran as a closely spaced doublet (18). RT-PCR analysis has shown that the upper isoform represents the ortholog to mouse *Foxp3*, whereas the lower isoform is encoded from an mRNA lacking exon 2 (amino acids 71–105) (76). This splicing variant has not been reported in mouse CD4⁺CD25⁺ Tregs. Whether the same T cell expresses both isoforms simultaneously is currently not known, although all sources of FOXP3 in humans have both isoforms. In addition, it is unclear if there is a functional difference between the two isoforms. However, expression of the Δ exon2 isoform in CD4⁺CD25⁻FOXP3⁻ human T cells leads to functional anergy, but not to the same extent as expression of full-length FOXP3 (76). Human T cells expressing FOXP3 Δ exon2 have intermediate proliferative responses to TCR stimulation and make slightly more IL-2 than cells expressing only the full length. Interestingly, the sequences encoded by exon 2 fall within one of the functional domains of FOXP3 defined above. Thus, it is possible that the lower isoform represents a natural dominant-negative form of FOXP3. Our lab has recently shown that FOXP3 and the orphan retinoic acid nuclear receptor ROR α interact and that the region of FOXP3 involved in the interaction is encoded by exon 2 (J. Du & S.F. Ziegler, manuscript in preparation). We are currently characterizing the molecular basis for this interaction.

Another clue that the regulation of *Foxp3* expression was not conserved between humans and mice comes from an analysis of FOXP3 expression in stimulated human CD4⁺CD25⁻ T cells. The starting population of T cells lacked detectable FOXP3 expression, but within 24 h of stimulation anti-CD3⁺CD28 FOXP3 expression was detected, peaking at 72 h (18). Similar experiments using mouse CD4⁺CD25⁻ T cells did not

demonstrate upregulation of *Foxp3* (15, 16). Further analysis with human CD4⁺ T cells showed that the subset of T cells that up-regulated CD25 also expressed FOXP3 (18). Thus, in humans, *FOXP3* was behaving like an activation-induced gene in CD4⁺ cells. These initial findings have been confirmed by other groups (76–78) and provide the first clue that human and mouse *Foxp3* are regulated differently. Consistent with the idea that FOXP3 expression in human CD4⁺ T cells is linked to TCR-mediated stimulation, CD4⁺ human T cell clones are also FOXP3⁺ (77–79; J.H. Buckner & S.F. Ziegler, unpublished observations). Mouse T cell clones have not been found to express *Foxp3*.

As described earlier, ectopic expression of *Foxp3* in conventional mouse CD4⁺ T cells converted those cells to Tregs (15, 16). Several groups have now attempted similar experiments with human FOXP3 using either retroviral or Lentiviral transduction of CD4⁺CD25⁻ T cells. As is seen in the mouse system, the transduced cells display anergy when stimulated through the TCR and are capable of suppressing responder CD4⁺ T cells (76, 80, 81). However, the level of suppression is not at the levels seen in the mouse experiments. Also, these experiments are somewhat confounded by the fact that the CD4⁺ T cells are stimulated prior to infection, which induces the expression of endogenous *FOXP3* (18, 76, 77).

As described above, stimulation of human CD4⁺CD25⁻ T cells leads to *FOXP3* expression. An obvious question is what is the functional outcome for the CD4⁺ T cell that induces FOXP3 expression? Recent work from our group has shown that these cells develop the capability to act as Tregs (18). When the CD25⁺ cells are isolated following the 10-day culture period, they have the ability to suppress the proliferation of freshly isolated responder T cells. One possible explanation for this phenomenon is that a preexisting pool of CD4⁺CD25⁻FOXP3⁺ cells preferentially expands in the cultures. However, this does not appear to be the case.

There is no detectable FOXP3 expression in the starting population of cells. In addition, when the CD4⁺CD25⁻ cells, isolated after the 10-day culture period, are restimulated, the same ratio of CD25⁺FOXP3⁺/CD25⁻FOXP3⁻ cells results (M.R. Walker, P. Putheti, J.H. Buckner, and S.F. Ziegler, unpublished data). Further support comes from the finding that naive CD4⁺ T cells (CD45RA⁺) and CD4⁺CD25⁻ T cells from cord blood are also capable of inducing FOXP3 and becoming Tregs, suggesting that antigen experience is not required for this process (82). Taken as a whole, these data support a model in which the ability of a CD4⁺ T cell to induce FOXP3 expression is not a developmentally determined event. Rather, it is dependent on the conditions present at the time of antigen stimulation of a given T cell. The underlying molecular mechanism that governs this development remains to be determined.

Another piece of evidence regarding the ability to generate Tregs in vitro using human CD4⁺ T cells comes from studies using specific antigens, rather than antibody crosslinking, as stimulus. For example, Walker et al. (82) showed that coculture of CD4⁺CD25⁻ T cells with allo-antigen-presenting cells (APCs) resulted in the development of Tregs specific for the stimulating allo-APCs. Although these experiments support a model of de novo Treg generation, the actual stimulating antigen is not known. However, they do demonstrate the specificity of the in vitro-generated Tregs in that they were unable to suppress responses stimulated by third-party APCs.

The final piece of evidence that induction of FOXP3 expression and de novo Treg generation can occur following antigen stimulation comes from studies using human MHC class II tetramers to isolate antigen-specific cells following in vitro stimulation. CD4⁺CD25⁻ T cells from DR*0401 (DR4) individuals were stimulated with the immunodominant peptide from influenza virus, HA(306–319), in the presence of ir-

radiated APCs. After 10 days of culture, tetramer⁺CD25⁺ cells were isolated from the cultures. These cells expressed FOXP3 and inhibited the subsequent proliferation of freshly isolated responder CD4⁺ T cells from the same donor (82). However, these HA(306–319)⁺CD25⁺FOXP3⁺ T cells did not affect the proliferation of T cells when stimulated with an unrelated antigen, tetanus toxoid, unless they also were given the HA peptide. Thus, they required cognate antigen for activation, but once activated they were capable of suppressing responses in an antigen-nonspecific manner.

The data outlined above suggest that there are at least two distinct populations of Tregs in humans. One population is generated in the thymus, is self-reactive, and is involved in protection from autoimmune responses. These Tregs are referred to as natural Tregs and would be analogous to those seen in the mouse that arise during thymic selection (83). The role of this population of Tregs is to protect against self-reactive effector T cells in the periphery. These cells comprise the CD4⁺CD25⁺ T cell population in human peripheral blood and are equivalent to those Tregs described in the mouse. This population of Tregs is also likely to be most affected by the mutations in *FOXP3* seen in IPEX patients. These mutations can be expected to result either in the development of self-reactive T cells that lack suppressor activity or in the complete lack of this population altogether. In either instance, the result would be a failure in the ability to control self-reactive effector T cells. In this respect, the IPEX patients resemble the *scurfy* mice.

A second population of Tregs is represented by CD4⁺CD25⁺FOXP3⁺ cells that are generated upon in vitro stimulation of CD4⁺CD25⁻FOXP3⁻ cells. By all criteria measured, these cells are indistinguishable from natural Tregs (18, 82; J.H. Buckner & S.F. Ziegler, unpublished data). As described above, they can be generated following polyclonal or antigen-specific stimulation. Also, similar to natural Tregs, their ability to

suppress is IL-10 and TGF- β independent, but is contact dependent (18). In addition, transcript array analysis has shown that the same set of genes regulated in natural Tregs appears to be similarly regulated in the *in vitro*-generated cells (P. Putheti & S.F. Ziegler, unpublished data). Taken together, the data demonstrate that CD4⁺ T cells with the same functional properties as thymically derived Tregs can be generated from naive conventional CD4⁺ T cells following TCR stimulation. Although the *in vivo* function of these cells is not known, we propose that they play a role in controlling immune responses, possibly serving to limit bystander activation at the site of the response. In addition, there are reports that CD4⁺CD25⁺ Tregs can induce infectious tolerance through the local generation of classes of regulatory T cells (e.g., Tr1 cells) (84), further extending this paradigm. An unanswered question at this point is the fate of the induced Tregs. Because these cells appear to respond poorly to proliferative signals and are impaired in cytokine production, it seems likely that they will not persist once the response has ended and antigen is no longer available.

FOXP3 AND THERAPEUTIC INTERVENTION FOR AUTOIMMUNITY

As described above, following retroviral-mediated introduction of Foxp3, conventional CD4⁺ T cells acquire a regulatory-like phenotype and are capable of suppressing immune responses both *in vitro* and *in vivo* (15–17). The *in vivo* experiments involved cotransfer of Foxp3-transduced T cells with pathogenic CD4⁺ T cells in a model of colitis. These studies suggest the possibility of using ectopic Foxp3 expression to convert T cells to take on a regulatory phenotype and then using these cells in adoptive cellular immunotherapy. Coupled with the ability to identify and isolate antigen-specific human CD4⁺ T cells using MHC class II tetramers (85–87), *ex vivo* generation of antigen-specific

Tregs has great promise. Indeed, some recent reports have suggested that this approach may be viable. For example, Jaeckel et al. (27) used Foxp3-transduced T cells from BDC2.5 TCR transgenic mice to treat NOD mice with recent onset diabetes. They found that the antigen-specific BDC2.5 T cells, expressing Foxp3, were capable of reversing disease. However, they also showed that transfer of Foxp3-transduced polyclonal T cells from NOD mice failed to rescue. These data suggest that the critical feature for this type of therapy may be getting enough antigen-specific Tregs to the correct site, and that the transduced polyclonal T cells did not contain sufficient islet-antigen-specific cells to have a therapeutic effect.

In contrast to that study, Loser et al. (88) have shown that Foxp3-transduced polyclonal naive T cells, when transferred to sensitized hosts, could inhibit contact hypersensitivity responses. These polyclonal T cells were also used to treat mice expressing a keratin 14-CD40L transgene. These mice develop chronic skin inflammation and systemic autoimmunity (89). Transfer of Foxp3-transduced CD4⁺ T cells into these animals protected against both the local skin inflammation and the systemic autoimmunity (88).

Transferring these types of therapies to humans presents unique problems. As described above, stimulation of human CD4⁺CD25⁻ T cells induces FOXP3 expression, and most methods for introducing foreign genes into T cells using viral vectors require some level of stimulation. Thus, the very cells one may want to transduce and convert to Tregs may be less likely to be infected owing to expression of FOXP3 and a lowered overall activation level. However, the work from Walker et al. (82) suggests that deriving antigen-specific CD4⁺ T cells *in vitro* with self-antigens, followed by isolation of CD25⁺ that are also MHC class II tetramer⁺, may be a solution to this problem. The obvious limitation to this approach is the availability of the appropriate MHC class II tetramer for a given HLA haplotype and specific epitope.

In summary, the reemergence of suppressor T cells and the discovery of FOXP3 as a critical player in their development and function hold great promise for the treatment of autoimmune diseases in humans. The differences between humans and mice may present

obstacles in the transfer from mouse models to actual human disease. Thus, a more concerted effort to develop better human in vitro and in vivo systems is required to understand better the potential therapeutic benefits of manipulating FOXP3.

ACKNOWLEDGMENTS

The author thanks Drs. Megan K. Levings (University of British Columbia) and Troy R. Torgerson (University of Washington) for sharing information and data prior to publication, and Drs. Jane H. Buckner, Daniel J. Campbell, and Gerald T. Nepom (Benaroya Research Institute) for critical reading of the manuscript prior to submission. The author is supported by grants from the NIH (AI48779, AI059926, DK068312), American Diabetes Association, Juvenile Diabetes Research Foundation, and DANA Foundation.

LITERATURE CITED

1. Baecher-Allan C, Viglietta V, Hafler DA. 2004. Human CD4⁺CD25⁺ regulatory T cells. *Semin. Immunol.* 16:89–98
2. Piccirillo CA, Thornton AM. 2004. Cornerstone of peripheral tolerance: naturally occurring CD4⁺CD25⁺ regulatory T cells. *Trends Immunol.* 25:374–80
3. Shevach EM. 2002. CD4⁺CD25⁺ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2:389–400
4. Sakaguchi S. 2005. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6:345–52
5. Fontenot JD, Rudensky AY. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat. Immunol.* 6:331–37
6. Godfrey V, Rouse BT, Wilkinson JE. 1994. Transplantation of T cell-mediated lymphoreticular disease from the scurfy (*sf*) mouse. *Am. J. Pathol.* 145:281–86
7. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, et al. 1995. Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4*. *Science* 270:985–88
8. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541–47
9. Kanangat S, Blair P, Reddy R, Deheshia M, Godfrey V, et al. 1996. Disease in the scurfy (*sf*) mouse is associated with overexpression of cytokine genes. *Eur. J. Immunol.* 26:161–65
10. Clark LB, Appleby MW, Brunkow ME, Wilkinson JE, Ziegler SF, Ramsdell F. 1999. Cellular and molecular characterization of the scurfy mouse mutant. *J. Immunol.* 162:2546–54
11. Bettelli E, Dastrange M, Oukka M. 2005. Foxp3 interacts with nuclear factor of activated T cells and NF-κB to repress cytokine gene expression and effector functions of T helper cells. *Proc. Natl. Acad. Sci. USA* 102:5138–43
12. Brunkow ME, Jeffery EW, Hjerrild KA, Paepers B, Clark LB, et al. 2001. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27:68–73
13. Khattry R, Kasprowicz DJ, Cox T, Yasayko S-A, Ziegler SF, Ramsdell F. 2001. The

12. This paper describes the identification of *Foxp3* as the gene mutated in scurfy mice and describes the transgenic animals used to study Foxp3's role in CD4 T cell function.

15, 16, and 17. These seminal papers demonstrate conclusively Foxp3's critical role in Treg development and function.

18. This was the first paper to show that, in humans, FOXP3 expression can be induced by TCR stimulation, that cells with regulatory capability can be generated de novo, and that regulation of human and mouse Foxp3 have key differences.

19. This paper "rediscovered" Tregs as a distinct population of cells. Much subsequent work on Tregs and control of peripheral tolerance derives from the work described here.

21 and 22. These two papers describe the Foxp3 reporter mice that are vital to the study of Tregs as, in the mouse, Foxp3 is the only marker that appears to be exclusive to these cells.

- amount of scurf protein determines peripheral T cell number and responsiveness. *J. Immunol.* 167:6312–20
- 13a. Kasprrowicz DJ, Droin N, Ramsdell F, Green DR, Ziegler SF. 2006. Elevated FoxP3 expression leads to activation-induced cell death, while effector cell differentiation correlates with loss of FoxP3 expression. *Eur J. Immunol.* In press
14. Kasprrowicz DJ, Smallwood PS, Tzgnik AJ, Ziegler SF. 2003. Scurfin (FoxP3) controls T-dependent immune responses in vivo through regulation of CD4⁺ T cell effector function. *J. Immunol.* 171:1216–23
15. Fontenot JD, Gavin MA, Rudensky AY. 2003. FoxP3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 4:330–36
16. Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor FoxP3. *Science* 299:1057–61
17. Khattri R, Cox T, Yasayko S-A, Ramsdell F. 2003. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 4:337–42
18. Walker MR, Kasprrowicz DJ, Gersuk VH, Benard A, Van Landeghen M, et al. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁻ T cells. *J. Clin. Invest.* 112:1437–43
19. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151–64
20. Suri-Payer E, Amar AZ, Thornton AM, Shevach EM. 1998. CD4⁺CD25⁺ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* 160:1212–18
21. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329–41
22. Wan YY, Flavell RA. 2005. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc. Natl. Acad. Sci. USA* 102:5126–31
23. Kohm AP, Carpentier PA, Anger HA, Miller SD. 2002. Cutting edge: CD4⁺CD25⁺ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* 169:4712–16
24. Wraith DC, Nicolson KS, Whitely NT. 2004. Regulatory CD4⁺ T cells and the control of autoimmune disease. *Curr. Opin. Immunol.* 16:695–701
25. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, et al. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* 199:1455–65
26. Chai JG, Xue SA, Coe D, Addey C, Bartok I, et al. 2005. Regulatory T cells, derived from naive CD4⁺CD25⁻ T cells by in vitro Foxp3 gene transfer, can induce transplantation tolerance. *Transplantation* 79:1310–16
27. Jaeckel E, von Boehmer H, Manns MP. 2005. Antigen-specific FoxP3-transduced T-cells can control established type 1 diabetes. *Diabetes* 54:306–10
28. Lundsgaard D, Holm TL, Hornum L, Markholst H. 2005. In vivo control of diabetogenic T-cells by regulatory CD4⁺CD25⁺ T-cells expressing Foxp3. *Diabetes* 54:1040–47
29. Tai X, Cowan M, Feigenbaum L, Singer A. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat. Immunol.* 6:152–62

30. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, et al. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4⁺CD28⁺ regulatory T cells. *J. Immunol.* 171:3348–52
31. Boden E, Tang Q, Bour-Jordan H, Bluestone JA. 2003. The role of CD28 and CTLA4 in the function and homeostasis of CD4⁺CD25⁺ regulatory T cells. *Novartis Found. Symp.* 252:55–63
32. Scheffold A, Hühn J, Höfer T. 2005. Regulation of CD4⁺CD25⁺ regulatory T cell activity: it takes (IL-)two to tango. *Eur. J. Immunol.* 35:1336–41
33. Nelson BH. 2004. IL-2, regulatory T cells, and tolerance. *J. Immunol.* 172:3983–88
34. Malek TR, Bayer AL. 2004. Tolerance, no immunity, critically depends on IL-2. *Nat. Rev. Immunol.* 4:665–74
35. Nelson BH. 2002. Interleukin-2 signaling and the maintenance of self-tolerance. *Curr. Dir. Autoimmun.* 5:92–112
36. Schimpl A, Berberich I, Kneitz B, Kramer S, Santner-Nanan B, et al. 2002. IL-2 and autoimmune disease. *Cytokine Growth Factor Rev.* 13:369–78
37. Snow JW, Abraham N, Ma MC, Herndier BG, Pastuszak AW, Goldsmith MA. 2003. Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice. *J. Immunol.* 171:5042–50
38. Burchill MA, Goetz CA, Prlic M, O'Neil JJ, Harmon IR, et al. 2003. Distinct effects of STAT5 activation on CD4⁺ and CD8⁺ T cell homeostasis: development of CD4⁺CD25⁺ regulatory T cells versus CD8⁺ memory T cells. *J. Immunol.* 171:5853–64
39. Malek TR, Porter BO, Codias EK, Scibelli P, Yu A. 2000. Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. *J. Immunol.* 164:2905–14
40. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2R β -deficient mice: implications for the nonredundant function of IL-2. *Immunity* 17:167–78
41. Bayer AL, Yu A, Adeegbe D, Malek TR. 2005. Essential role for interleukin-2 for CD4⁺CD25⁺ T regulatory cell development during the neonatal period. *J. Exp. Med.* 201:769–77
42. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. 2005. Homeostatic maintenance of natural Foxp3⁺CD25⁺CD4⁺ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201:723–35
43. Marie JC, Letterio JJ, Gavin M, Rudensky AY. 2005. TGF- β 1 maintains suppressor function and Foxp3 expression in CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* 201:1061–67
44. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. 2004. TGF- β regulates in vivo expansion of Foxp3-expressing CD4⁺CD25⁺ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. USA* 101:4572–77
45. Chen W, Jin W, Hardegen N, Lei KJ, Li L, et al. 2003. Conversion of peripheral CD4⁺CD25⁻ T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor FoxP3. *J. Exp. Med.* 198:1875–86
46. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. 2004. Cutting edge: TGF- β induces a regulatory phenotype in CD4⁺CD25⁻ T cells through FoxP3 induction and down-regulation of Smad7. *J. Immunol.* 172:5149–53
47. Fu S, Zhang N, Yopp AC, Chen D, Mao M, et al. 2004. TGF- β induces Foxp3⁺ T-regulatory cells from CD4⁺CD25⁻ precursors. *Am. J. Transplant.* 4:1614–27
48. Nelson BH, Martyrak TP, Thompson LJ, Moon JJ, Wang T. 2003. Uncoupling of

- promitogenic and antiapoptotic functions of IL-2 by Smad-dependent TGF- β signaling. *J. Immunol.* 170:5563–70
49. Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, et al. 2002. CD4⁺CD25⁺ regulatory T cells can mediate suppressor function in the absence of transforming growth factor β 1 production and responsiveness. *J. Exp. Med.* 196:237–46
 50. Schramm C, Huber S, Protschka M, Czochra P, Burg J, et al. 2004. TGF β regulates the CD4⁺CD25⁺ T-cell pool and the expression of Foxp3 in vivo. *Int. Immunol.* 16:1241–49
 51. Huber S, Schramm C, Lehr HA, Mann A, Schmitt S, et al. 2004. Cutting edge: TGF- β signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4⁺CD25⁺ T cells. *J. Immunol.* 173:6526–31
 52. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman R, et al. 2005. T cells that cannot respond to TGF- β escape control by CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* 201:737–46
 53. Kaestner K, Knöchel W, Martinez D. 2000. Unified nomenclature for the winged-helix/forkhead transcription factors. *Genes Dev.* 14:142–46
 54. Kaufmann E, Knöchel W. 1996. Five years on the wings of fork head. *Mech. Dev.* 57:3–20
 55. Kaufmann E, Müller D, Knöchel W. 1995. DNA recognition site analysis of *Xenopus* winged helix proteins. *J. Mol. Biol.* 248:239–54
 56. Li C, Tucker PW. 1993. DNA-binding properties and secondary structural model of the hepatocyte nuclear factor 3/fork head domain. *Proc. Natl. Acad. Sci. USA* 90:11583–87
 57. Schubert LA, Jeffery EW, Zhang Y, Ramsdell F, Ziegler SF. 2001. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J. Biol. Chem.* 276:37672–79
 58. Li S, Weidenfeld J, Morrisey EE. 2004. Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. *Mol. Cell. Biol.* 24:809–22
 59. Wang B, Lin D, Li C, Tucker PW. 2003. Multiple domains define the expression and regulatory properties of Foxp1 forkhead transcriptional repressors. *J. Biol. Chem.* 278:24259–68
 60. Powell BR, Buist N, Stenzel P. 1982. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. *J. Pediatr.* 100:731–37
 61. Bennett CL, Ochs HD. 2001. IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. *Curr. Opin. Pediatr.* 13:533–38
 62. Gambineri E, Torgerson TR, Ochs HD. 2003. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of *FOXP3*, a critical regulator of T cell homeostasis. *Curr. Opin. Rheumatol.* 15:430–35
 63. Torgerson TR, Ochs HD. 2002. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: a model of immune dysregulation. *Curr. Opin. Allergy Clin. Immunol.* 2:481–87
 64. Nieves DS, Phipps RP, Pollock SJ, Ochs HD, Zhu Q, et al. 2004. Dermatologic and immunologic findings in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Arch. Dermatol.* 140:466–72
 65. Ochs HD, Ziegler SF, Torgerson TR. 2005. FOXP3 acts as a rheostat of the immune response. *Immunol. Rev.* 203:156–64
 66. Owen CJ, Jennings CE, Imrie H, Lachaux A, Bridges NA, et al. 2003. Mutational analysis

- of the FOXP3 gene and evidence for genetic heterogeneity in the immunodysregulation, polyendocrinopathy, enteropathy syndrome. *J. Clin. Endocrinol. Metab.* 88:6034–39
67. Kobayashi I, Shiari R, Yamada M, Kawamura N, Okano M, et al. 2001. Novel mutations of FOXP3 in two Japanese patients with immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome (IPEX). *J. Med. Genet.* 38:874–76
 68. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova J-L, et al. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 27:18–20
 69. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PF, et al. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutation of FOXP3. *Nat. Genet.* 27:20–21
 70. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, et al. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J. Clin. Invest.* 106:R75–81
 71. Wilden RS, Smyk-Pearson S, Filipovich AH. 2002. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy X linked (IPEX) syndrome. *J. Med. Genet.* 39:537–45
 72. Jonuleit H, Schmitt E, Stassen M, Tüttenberg A, Knop J, Enk AH. 2001. Identification and functional characterization of human CD4⁺CD25⁺ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193:1285–94
 73. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. 2001. Ex vivo isolation and characterization of CD4⁺CD25⁺ T cells with regulatory properties from human blood. *J. Exp. Med.* 193:1303–10
 74. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. 2001. CD4⁺CD25^{high} regulatory cells in human peripheral blood. *J. Immunol.* 167:1245–53
 75. Levings MK, Sangregorio R, Roncarolo MG. 2001. Human CD25⁺CD4⁺ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J. Exp. Med.* 193:1295–302
 76. Allan SE, Passerini L, Bacchetta R, Crellin N, Da M, et al. 2006. The role of FOXP3, and an isoform lacking exon 2, in the generation of human CD4⁺ T regulatory cells. *J. Clin. Invest.* In press
 77. Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, et al. 2005. Expression of FOXP3 mRNA is not confined to CD4⁺CD25⁺ T regulatory cells in humans. *Hum. Immunol.* 66:13–20
 78. Roncador G, Brown PJ, Maestre L, Hue S, Martinex-Torrecaudrada JL, et al. 2005. Analysis of FOXP3 protein expression in human CD4⁺CD25⁺ regulatory T cells at the single-cell level. *Eur. J. Immunol.* 18:1681–91
 79. Malone R, Kochik SA, Reijonen H, Carson BD, Ziegler SF, et al. 2006. Functional avidity directs T-cell fate in autoreactive CD4⁺ T-cells. *Blood.* In press
 80. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, et al. 2004. Crucial role of FOXP3 in the development and function of human CD25⁺CD4⁺ regulatory T cells. *Int. Immunol.* 16:1643–56
 81. Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, et al. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T cells. *PLoS Biol.* 2:955–66
 82. Walker MR, Carson BD, Nepom GT, Ziegler SF, Buckner JH. 2005. De novo generation of antigen-specific CD4⁺CD25⁺ regulatory T cells from human CD4⁺. *Proc. Natl. Acad. Sci. USA* 102:4103–8

83. Bluestone JA, Abbas AK. 2003. Natural versus adapted regulatory T cells. *Nat. Rev. Immunol.* 3:253–57
84. Moore KJ, de Waal MR, Coffman R, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19:683–765
85. Novak EJ, Masewics SA, Liu AW, Lernmark A, Kwok WW, Nepom GT. 2001. Activated human epitope-specific T cells identified by class II tetramers reside within a CD4^{high}, proliferating subset. *Int. Immunol.* 13:799–806
86. Buckner JH, Holzer U, Novak EJ, Reijonen H, Kwok WW, Nepom GT. 2002. Defining antigen-specific responses with human MHC class II tetramers. *J. Allergy. Clin. Immunol.* 110:199–208
87. Nepom GT, Buckner JH, Novak EJ, Reichstetter S, Reijonen H, et al. 2002. HLA class II tetramers. Tools for direct analysis of antigen-specific CD4⁺ T cells. *Arthritis Rheum.* 46:5–12
88. Loser K, Hansen W, Apelt J, Balkow S, Buer J, Beissert S. 2005. In vitro-generated regulatory T cells induced by FoxP3-retrovirus infection control murine contact allergy and systemic autoimmunity. *Gene Ther.* In press
89. Mehling A, Loser K, Varga G, Metz D, Luger TA, et al. 2001. Overexpression of CD40 ligand in murine epidermis results in chronic skin inflammation and systemic autoimmunity. *J. Exp. Med.* 194:615–28
90. Cofffer PJ, Burgering BM. 2004. Forkhead box transcription factors and their role in the immune system. *Nat. Rev. Immunol.* 4:889–99
91. Pignata C, Gaetaniello L, Masci AM, Frank J, Christiano A, et al. 2001. Human equivalent of the mouse Nude/SCID phenotype: long-term evaluation of immunologic reconstitution after bone marrow transplantation. *Blood* 97:880–85
92. Nehls M, Pfeifer D, Schorpp M, Hedrich H, Boehm T. 1994. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372:103–7
93. Lin L, Spoor MS, Gerth AJ, Body SL, Peng SL. 2004. Modulation of Th1 activation and inflammation by the NF- κ B repressor Foxj1. *Science* 303:1017–20
94. Lin L, Hron JD, Peng SL. 2004. Regulation of NF- κ B, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* 21:203–13
95. Burgering BM, Kops GJ. 2002. Cell cycle and death control: long live Forkheads. *Trends Biochem. Sci.* 27:352–60



Contents

Frontispiece <i>Jack L. Strominger</i>	x
The Tortuous Journey of a Biochemist to Immunoland and What He Found There <i>Jack L. Strominger</i>	1
Osteoimmunology: Interplay Between the Immune System and Bone Metabolism <i>Matthew C. Walsh, Nacksung Kim, Yubo Kadono, Jaerang Rho, Soo Young Lee, Joseph Lorenzo, and Yongwon Choi</i>	33
A Molecular Perspective of CTLA-4 Function <i>Wendy A. Teft, Mark G. Kirchhof, and Joaquín Madrenas</i>	65
Transforming Growth Factor- β Regulation of Immune Responses <i>Ming O. Li, Yisong Y. Wan, Shomyeh Sanjabi, Anna-Karin L. Robertson, and Richard A. Flavell</i>	99
The Eosinophil <i>Marc E. Rothenberg and Simon P. Hogan</i>	147
Human T Cell Responses Against Melanoma <i>Thierry Boon, Pierre G. Coulie, Benoît J. Van den Eynde, and Pierre van der Bruggen</i>	175
FOXP3: Of Mice and Men <i>Steven F. Ziegler</i>	209
HIV Vaccines <i>Andrew J. McMichael</i>	227
Natural Killer Cell Developmental Pathways: A Question of Balance <i>James P. Di Santo</i>	257
Development of Human Lymphoid Cells <i>Bianca Blom and Hergen Spits</i>	287
Genetic Disorders of Programmed Cell Death in the Immune System <i>Nicolas Bidère, Helen C. Su, and Michael J. Lenardo</i>	321

Genetic Analysis of Host Resistance: Toll-Like Receptor Signaling and Immunity at Large <i>Bruce Beutler, Zhengfan Jiang, Philippe Georgel, Karine Crozat, Ben Croker, Sophie Rutschmann, Xin Du, and Kasper Hoebe</i>	353
Multiplexed Protein Array Platforms for Analysis of Autoimmune Diseases <i>Imelda Balboni, Steven M. Chan, Michael Kattab, Jessica D. Tenenbaum, Atul J. Butte, and Paul J. Utz</i>	391
How TCRs Bind MHCs, Peptides, and Coreceptors <i>Markus G. Rudolph, Robyn L. Stanfield, and Ian A. Wilson</i>	419
B Cell Immunobiology in Disease: Evolving Concepts from the Clinic <i>Flavius Martin and Andrew C. Chan</i>	467
The Evolution of Adaptive Immunity <i>Zeev Pancer and Max D. Cooper</i>	497
Cooperation Between CD4 ⁺ and CD8 ⁺ T Cells: When, Where, and How <i>Flora Castellino and Ronald N. Germain</i>	519
Mechanism and Control of V(D)J Recombination at the Immunoglobulin Heavy Chain Locus <i>David Jung, Cosmas Giallourakis, Raul Mostoslavsky, and Frederick W. Alt</i>	541
A Central Role for Central Tolerance <i>Bruno Kyewski and Ludger Klein</i>	571
Regulation of Th2 Differentiation and <i>Il4</i> Locus Accessibility <i>K. Mark Ansel, Ivana Djuretic, Bogdan Tanasa, and Anjana Rao</i>	607
Diverse Functions of IL-2, IL-15, and IL-7 in Lymphoid Homeostasis <i>Averil Ma, Rima Koka, and Patrick Burkett</i>	657
Intestinal and Pulmonary Mucosal T Cells: Local Heroes Fight to Maintain the Status Quo <i>Leo Lefrançois and Lynn Puddington</i>	681
Determinants of Lymphoid-Myeloid Lineage Diversification <i>Catherine V. Laïosa, Matthias Stadtfeld, and Thomas Graf</i>	705
GP120: Target for Neutralizing HIV-1 Antibodies <i>Ralph Pantophlet and Dennis R. Burton</i>	739
Compartmentalized Ras/MAPK Signaling <i>Adam Mor and Mark R. Philips</i>	771