Minireview

FOXP3 and NFAT: Partners in Tolerance

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Regulatory T cells suppress autoimmune responses to self-antigens. Recent studies, including one in this issue of Cell (Wu et al., 2006), suggest that the ability of T cells to choose between launching a productive immune response, functional inactivation, or developing into regulatory T cells depends upon the interplay of the key transcriptional regulators FOXP3 and NFAT.

The immune system of higher organisms is faced with the daily challenge of distinguishing between foreign and “self” antigens. Part of this challenge involves recognizing the molecular features of an environment that an organism is chronically exposed to (such as products of commensal flora) in addition to its own molecular components. Furthermore, this “self” antigenic landscape evolves during the life span of an organism because of the expression of temporarily regulated genes (e.g., during metamorphosis or sexual maturation) and of a changing microbial and nonmicrobial environment. Thus, it is a daunting task to evolve a regulatory mechanism that allows the immune system to efficiently protect against infection while avoiding destruction of the organism by lymphocytes bearing receptors specific for self-antigens. Avoiding self-reactivity is particularly challenging for T cells whose antigen receptors (TCRs) recognize short peptides bound to MHC gene products. T cells recognize peptide-MHC complexes on antigen-presenting cells that are generated during continuous turnover of endogenously synthesized and internalized proteins. The overwhelming majority of MHC bound peptides are derived from self proteins because the antigen-processing machinery and MHC molecules themselves do not discriminate between self or foreign protein products. To make matters even more complicated, certain low-avidity interactions of TCRs with self-peptide-MHC complexes are necessary for the successful maturation of T cells in the thymus and for their survival in the periphery.

One obstacle to the spurious activation of naive peripheral T cells upon TCR recognition of self-ligands is a requirement for an additional signal. This signal emanates from a costimulatory activating receptor, such as CD28, for a productive immune response to proceed. The costimulatory molecules CD80 and CD86 serve as CD28 ligands and are upregulated in antigen-presenting cells upon exposure to microbial products that are recognized by a set of evolutionarily conserved pattern recognition receptors (Janeway, 1989; Medzhitov and Janeway, 1999). The immunological self/non-self-discrimination based on the two-signal requirement is complemented by mechanisms of immunological tolerance operating in a cell-intrinsic (recessive) and cell-extrinsic (dominant) fashion (described below). In this issue of Cell, Wu et al. (2006) suggest a connection between the transcriptional mechanisms that facilitate recessive and dominant tolerance.

Recessive Tolerance: Clonal Deletion and Anergy

During their development in the thymus, the majority of thymocytes with TCRs that exhibit high avidity for self-peptide-MHC complexes undergo programmed cell death resulting in the deletion of autoreactive clones. Those high-affinity self-reactive thymocytes that escape deletion acquire a state of unresponsiveness to TCR stimulation known as anergy. These mechanisms constitute recessive T cell tolerance. Recessive tolerance can also be induced after mature thymocytes exit the thymus as peripheral T cells subjected to chronic TCR stimulation by high-affinity ligands undergo deletion or become anergic. Recent studies revealed that the induction of anergy is an active process, and, at least in mature peripheral T cells, anergy can be elicited by sustained Ca2+ signaling caused by TCR induction. Ca2+ signaling leads to activation of members of the NFAT family of transcription factors in the absence of their interacting partner AP-1 (the Fos-Jun heterodimer) (Macian et al., 2002). In peripheral T cells, AP-1 is induced by CD28-mediated signals, and productive immune responses are dependent upon the cooperative binding of the NFAT/AP-1 transcriptional complex to the corresponding sequence elements within the promoter regions of certain genes. These genes encode factors involved in the immune response including IL-2, a major T cell growth factor, and IL-2 receptor α chain (CD25). In contrast, a distinct transcriptional program of anergy executed by NFAT in the absence of AP-1 leads to inability of T cells to mount a proliferative response to TCR engagement, to produce IL-2, as well as to differentiate into effector cells producing cytokines such as IFN-γ or TNF-α. Instead, anergized T cells express increased levels of IL-10, a potent anti-inflammatory cytokine. The anergic state in T cells is characterized by a sharp increase in the threshold of TCR activation and is actively maintained by continuous TCR stimulation. Providing exogenous IL-2...
and strong TCR/CD28 stimulation can break anergy and result in productive activation of anergic T cells. In this regard, anergy induction in a very broad sense can be considered as a special case of cell-intrinsic negative feedback regulation.

**Dominant Tolerance Mediated by Regulatory T Cells: A Key Role for FOXP3**

The aforementioned recessive tolerance mechanisms are complemented by a mechanism of dominant (cell-extrinsic) regulation of overexuberant immune responses mediated by a specialized subset of suppressive CD4 T lymphocytes. These cells, euphemistically dubbed regulatory T (Treg) cells, keep in check self-reactive T cells by poorly understood suppressive mechanisms. Treg cells, initially characterized by high level of surface expression of CD25, normally develop in the thymus and constitute ~7%–15% of peripheral CD4 T cells. Treg cells appear to exhibit some characteristic features of anergy as they fail to efficiently flux Ca^{2+}, proliferate on their own, or produce IL-2 as well as proinflammatory cytokines in response to TCR stimulation (see for review Sakaguchi, 2005).

Recently, the X chromosome-encoded transcription factor FOXP3 was shown to be expressed exclusively in Treg cells and required for their development in the thymus (see for review Ramsdell, 2003; Fontenot and Rudensky, 2005). Furthermore, in peripheral T cells, forced expression of a Foxp3 transgene resulted in acquisition of suppressive function. FOXP3 was originally cloned as a gene mutated in patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome and in the spontaneous mouse mutant scurfy. In IPEX patients, multiple mutations in the FOXP3 DNA binding forkhead domain (FKH) and leucine zipper have been reported (see for review Ramsdell, 2003; Ochs et al., 2005). Both in humans and mice, Foxp3 mutations result in fatal aggressive autoimmune pathology affecting multiple organs. Importantly, an identical phenotype was observed in mice in which a conditional Foxp3 allele was ablated specifically in the germline and the T cell lineage. This result suggests that a Foxp3 lesion in T cells is entirely responsible for the pathology associated with FOXP3 deficiency (Fontenot et al., 2005b). The latter observation—combined with the lack of a detectable role for Foxp3 in recessive tolerance or in the regulation of proliferative response and cytokine production by peripheral T cells—showed that the breakdown of dominant tolerance, i.e., lack of the Treg cells, results in early-onset, highly aggressive fatal autoimmunity. Thus, FOXP3 is a principal and dedicated mediator of the genetic mechanism of dominant tolerance (Fontenot et al., 2005b).

Transcriptional profiling of Treg cells expressing FOXP3 in comparison to naive or activated T cells revealed a substantial number of differentially expressed genes. Relatively few mRNAs are downregulated whereas expression of the majority of genes is increased. The latter group includes some of the genes normally upregulated in activated T cells, such as Il2ra (CD25), Ctla4 (CTLA-4), and Tnfsf18 (GITR). In agreement with the functional data, the IL-2 message was repressed in Treg cells when compared to activated T cells (see for review Sakaguchi, 2005; Fontenot and Rudensky, 2005). Although the FOXP3-dependent genetic program has remained largely unknown, Il2ra and Il2 have become prototype target genes for FOXP3. Indeed, it was shown recently that FOXP3-mediated repression of NFAT-dependent transcription occurs upon FOXP3 binding to a consensus forkhead binding motif that overlaps with the AP-1 site within the NFAT/AP-1 binding DNA-regulatory element. Thus, it was proposed that FOXP3 may compete with NFAT/AP-1 complexes for IL2 regulatory elements (Ziegler, 2006). These early experiments were corroborated and further extended by FOXP3 overexpression studies using retroviral transduction of activated T cells (Bettelli et al., 2005; Grant et al., 2006). The latter experiments also suggested that FOXP3 might also repress NF-κB and CREB transcriptional targets in activated T cells. Transient and stable transfection studies indicated that in addition to DNA binding FKH domain, FOXP3 protein–protein interaction domains, a unique proline-rich N-terminal region, and a leucine-zipper dimerization domain are required for FOXP3 function (Ziegler, 2006; Wu et al., 2006). A substantial caveat to these studies is that FOXP3 was overexpressed in cells that may lack FOXP3 cofactors or regulating signals. However, these results are consistent with the biochemical and structural analyses of the binding of other FOXP family members to DNA, which revealed a somewhat low affinity for DNA, suggesting a need for dimerization and interacting partners to facilitate FOXP3 transcriptional activity (Li et al., 2004; Stroud et al., 2006).

**A Mechanistic Link between Transcriptional Control of Recessive and Dominant Tolerance**

These observations raised a question of a possible role for NFAT not only in facilitating recessive tolerance via its role in anergy induction but also in dominant tolerance via its cooperation with FOXP3. Wu et al. (2006) now produce experimental evidence in support of this possibility by demonstrating cooperative binding of NFAT and FOXP3 to DNA. By solving the structure of NFAT and the related FOXP2 FKH domain bound to DNA, this study also provides details of the molecular interactions in this ternary complex (Wu et al., 2006). The latter experiments led to realization that structural requirements for NFAT interaction with AP-1 and FKH domain transcription factors are distinct, providing potential means to manipulate these interactions in the future using site-specific chemical inhibitors. More importantly, based on the structure, the authors mutated residues in the predicted NFAT interaction sites within the FOXP3 FKH domain to create a compound WWRR FOXP3 mutant (T359W N361W E399R E401R). This mutant lacks the ability to repress Il2 transcription upon retroviral transduction of activated T cells. Moreover, T cells transduced with the WWRR FOXP3 mutant—compared to T cells transduced with wild-type FOXP3—showed a lower increase
in the expression of CD25 and GITR. This result suggests that NFAT:FOXP3 cooperatively bind to the regulatory elements of genes (such as those encoding CD25 and GITR) that have an altered expression in Treg cells. Indeed, in T cells transduced with FOXP3—and in Treg cells propagated in vitro in the presence of IL-2—both NFAT and FOXP3 seem to bind within the promoter regions of genes that encode IL-2, CTLA-4, and CD25 as illustrated by chromatin immunoprecipitation experiments (Wu et al., 2006). How do NFAT:FOXP3 complexes impart negative and positive regulation upon binding to the corresponding regulatory elements within the same cell? Further in-depth analysis of the molecular makeup of higher-order transcriptional complexes formed by FOXP3 in Treg cells will address this issue. Another intriguing observation in these experiments was that FOXP3 and NFAT binding to the promoters of the genes that encode CTLA-4, IL-2, and CD25 was detected upon stimulation of transduced or ex vivo isolated T cells by PMA and ionomycin or ionomycin alone, respectively. At face value, these results suggest that Ca\(^{2+}\) signaling is required to detect NFAT and FOXP3 binding to the aforementioned promoters. If these data can be extrapolated to regulation of gene expression in Treg cells in vivo, Ca\(^{2+}\) signaling-dependent regulation of the occupancy of the corresponding promoters by NFAT along with FOXP3 in activated versus resting Treg cell states may not translate into significant changes in expression of these genes at the protein level. This is because resting Treg cells have a uniformly high level of CD25 expression. It remains to be seen whether chronic TCR stimulation that Treg cells typically encounter is sufficient to sustain occupancy of regulatory regions of NFAT:FOXP3 target genes in vivo.

Biochemical studies by Wu and coauthors suggest that the genes that encode CTLA-4, CD25, and IL-2 serve as direct targets of an NFAT:FOXP3 complex in Treg cells. However, arguably one of the most intriguing questions was raised by the final set of functional experiments in which T cells specific for pancreatic antigens were transduced with the WVRRR FOXP3 mutant. These transduced cells failed to acquire suppressive function and failed to prevent the development of diabetes caused by the adoptive cotransfer of untransduced pancreatic antigen-specific T cells. In contrast, T cell transduced with wild-type FOXP3 protein conferred potent suppressive capacity (Wu et al., 2006). These data imply that NFAT:FOXP3 mediated regulation of a still mysterious mechanism of Treg-mediated suppression or of differentiation of Treg cells. It seems unlikely that high-level CD25 expression in Treg cells is directly involved in regulation of their suppressive function or differentiation as CD25-deficient Treg cells develop and are capable of suppression at least in vitro (Fontenot et al., 2005a). A potential role for CTLA-4 in mediating suppression remains controversial as the arguments exist both for and against a role for CTLA-4 as a nonredundant suppressive molecule (reviewed in Sakaguchi, 2005). Additionally, a biological significance of FOXP3-mediated repression of IL-2 gene expression in Treg cells and its relevance to their suppressive function are unclear. This leaves open a possibility of a yet unknown molecular mechanism of Treg development or suppressive function dependent upon NFAT:FOXP3 cooperation. Obviously, this argument depends upon a very specific effect of WRRR mutations, i.e., exclusive impairment of NFAT:FOXP3 cooperative binding, but not NFAT-independent binding to DNA either in partnership with other transcriptional regulators or in a cofactor-independent manner. Identification of NFAT-dependent and -independent transcriptional programs mediated by FOXP3 in Treg cells will be instrumental in clarifying these issues.

Models for Regulatory T Cell Development

The emerging model of a lineage choice between effector versus regulatory T cell differentiation determined upon NFAT partnership with AP-1 or FOXP3, with anergy resulting from failure of both differentiation pathways (i.e., AP-1 and FOXP3 induction), is appealing in its simplicity (see Figure 1; Wu et al., 2006). This model is likely applicable to the generation of Treg cells in the periphery in response to chronic exposure to suboptimal TCR stimulation as recently demonstrated for TCR transgenic T cells exposed to low amounts of the cognate ligand in vivo (Kretschmer et al., 2005). In addition to TCR triggering, recent in vitro studies suggested a role for high dose of TGF-β in FOXP3 induction in peripheral T cells (Figure 2; reviewed in Sakaguchi, 2005). However, the aforementioned scenario may be more complex if a CD28 signal is required for peripheral Treg generation (see below). As an additional note of caution, it is far from certain whether differentiation of peripheral polyclonal T cells specific for self or foreign antigens into FOXP3-expressing T cells...
contributes in a substantial way to the overall peripheral Treg cell population and whether this process is essential for the maintenance of immune homeostasis under physiologic condition or during infection.

In contrast, a critical role for thymic differentiation of Treg cells is strongly suggested by the autoimmunity typically observed in day 3 thymectomized mice (Sakaguchi, 2005). More recently, comparative analysis of TCR repertoires displayed by thymic and peripheral Treg cells indicated substantial similarity between these Treg subsets as opposed to the relatively small overlap between TCR repertoires displayed by thymic and peripheral nonregulatory T cells (Hsieh et al., 2006). Furthermore, recent analysis of Leishmania-specific Treg cells present in skin of infected mice revealed that none of these cells acquired FOXP3 expression upon encounter with the pathogen. Instead, essentially all these cells originate from pre-existing Treg cell population (Suffia et al., 2006). In agreement with the day 3 thymectomy studies, these results suggest that thymically generated FOXP3-expressing Treg cells predominantly contribute to functional Treg population in the periphery with some possible exceptions for specific microenvironments such as the tumor microenvironment.

Can the model of NFAT:FOXP3-dependent generation of Treg cells in the periphery be extended to the thymus? Consistent with the model, regulatory T cells developing in the thymus use TCR with an increased affinity for self-peptide-MHC complexes (normally subject to anergy induction) and this signal seems to be necessary for induction of FOXP3 expression (reviewed in Sakaguchi, 2005; Fontenot and Rudensky, 2005). However, thymic Treg development is dependent upon CD28 signals (Salomon et al., 2000; Tai et al., 2005) and, likely, on additional unknown and known factors such as STAT5 activation downstream of γc cytokine receptors (Figure 2; Fontenot et al., 2005a). Therefore, AP-1 and FOXP3 are probably coexpressed in regulatory T cells developing in the thymus, implying a more complicated scenario depending upon relative concentrations of NFAT, AP-1, and FOXP3 as well as other yet unidentified FOXP3 transcriptional partners and cofactors. Future dissection of temporal expression of these transcriptional regulators and their targets during thymic development of Treg cells and of signaling events affecting their expression will help to better understand the key mechanisms of recessive and dominant immunological tolerance.

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