Commentary

Dynamics of Suppressor T Cells: In Vivo Veritas

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Immunity in the absence of autoimmunity reflects "self–nonself" discrimination by the immune system. The search for mechanisms preventing autoimmunity or enabling "self" tolerance has been at the root of immunology as evidenced by ample conceptual framework, rhetoric, sophism, and experimental data. A full understanding of such mechanisms would facilitate a large number of therapeutic interventions with immunity that are too diverse to be discussed here.

Of special interest is a pathway to self tolerance that involves regulatory or suppressor cells (also known as T_{Reg} cells). Curiously, these cells exhibit an anergic phenotype, being unable to proliferate upon TCR ligation in culture, at the very same time that their suppressive activity is manifested. This article recalls some of the early experiments that hinted at the existence of these suppressor cells—now a topic of intense investigation in several laboratories—and considers their dynamic nature in vivo, which has been newly recognized in a series of recent publications (1–4). The new evidence shows that suppressor T cells have a considerable intermitotic lifespan in the absence of Ag (1, 2). In addition, studies in normal mice and analyses of Ag-presenting cell requirements reveal that T_{Reg}, instead of being anergic, are capable of substantial Ag-induced expansion in vivo, accompanied by increased suppressive activity (1–4). The new findings raise questions concerning concepts on the pathogenesis of autoimmunity in certain gene-deficient strains of mice such as IL-2 or IL-2 receptor (IL-2R)–deficient mice.

Recessive and Dominant Tolerance. There is a distinction between recessive and dominant mechanisms of tolerance. Recessive tolerance includes deletion of T cells and deletion or receptor editing of B cells, whereas dominant tolerance is defined as that which can be transferred by a subset of cells from a tolerant donor into an immunocompetent host. Although initially there was some superfluous polarizations regarding the relevance of recessive versus dominant mechanisms of tolerance (5, 6), both mechanisms appear to be essential for prevention of autoimmunity (for review see reference 7).

Conclusive evidence of recessive tolerance indicated by peptide–MHC complexes (8–11) or superantigens (5, 12) in T cells and by antigens in B cells (13–16) was obtained before that of dominant tolerance (1–10). Early indirect and occasionally irreproducible results did not help the course of dominant tolerance, and thus it is difficult to pinpoint data that began to convince the scientific community of its importance. Among those certainly were thymus transplant (17) and thymectomy (18) experiments, indicating that the thymus might have a role in dominant tolerance: embryonic allogeneic thymus transplants between birds were shown to induce donor-specific tolerance to a subsequent wing graft (17), whereas thymectomy in the neonatal period of mice resulted in autoimmunity (18). More conclusively, in the latter scenario, tolerance to self could be restored by transfer of $CD4^+CD25^+$ T cells (19), whereas in the former, at that time reproduced in mice, $CD4^+$ T cells could transfer tolerance into an immunocompetent host (20).

Characterization of Regulatory T Cells. Even though $CD4^+25^+$ and $CD4^+25^-$ regulatory T cells do exist (21–23), the CD25 marker was useful to establish some properties of polyclonal in comparison to Ag-specific regulatory T cells. Gene expression analysis revealed high expression of the receptors CTL4–4 and programmed death-1 (24, 25) by regulatory T cells. However, the best marker is perhaps the forkhead transcription factor FOXP3 (26–28), which is expressed at high levels in regulatory $CD25^+CD4^+$ T cells but not or only weakly transcribed in naive or recently activated T cells. FOXP3 is of special interest because humans and mice defective in FOXP3 lack regulatory T cells and suffer from autoimmunity (26, 27). Of interest is also the glucocorticoid-induced TNFR-related protein (GITR) (29, 30) whose ligation on $CD25^+$ T cells results in loss of suppressive activity.

In vitro analysis of polyclonal $CD25^+$ cells suggested that in terms of CD3-specific antibody- and Ag-induced proliferation such cells were anergic unless supplied with high doses of exogenous IL-2 (31). Ag-induced proliferation was analyzed with $CD25^+$ T cells from TCR transgenic mice in which an αβTCR composed of transgenic TCRβ and endogenous TCRα chains was apparently required to generate such cells in vivo, whereas the transgenic αβTCR could be used as a vehicle to activate suppression by antigenic stimulation in vitro. Suppressive activity was apparent because of inhibition of proliferation of naive T cells which were cocultured with regulatory T cells. Such inhibition required activation of the regulatory T cells through their TCR, direct cell contact between suppressor and suppressed T cells, and was independent of IL-10 or TGF-β

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production and/or interactions of these cytokines with their receptors. Suppression resulted in the inability of naive T cells to transcribe the IL-2 gene. Apart from these observations, the molecular nature of interactions that result in the suppression of proliferation by naive T cells has remained obscure (31).

The same can be said for suppressive activity in vivo except perhaps for the notion that in some models of autoimmune disease, such as inflammatory bowel disease precipitated by injecting naive $CD4^+25^-$ cells into lymphopenic hosts, amelioration of disease by regulatory T cells essentially depended on their production of IL-10 (32). Suppression of other forms of immunity, however, could proceed in the absence of IL-10 binding to its receptor (33). Studies too diverse to be listed here suggested that almost all manifestations of immunity could be diminished by regulatory T cells.

Origin of Regulatory T Cells. Transgenic mice coexpressing class II MHC-restricted TCRs and their agonist ligands have been useful in delineating some pathways of T_{Reg} generation. Initially, it was noted that such coexpression of TCR and ligand resulted in a much increased frequency of $CD25^+$ T_{Reg} with the transgenic TCR (34). Follow-up studies in TCR transgenic $RAG^{-/-}$ mice showed that the interaction of the TCR with its agonist ligand could result in $CD25^+$ T_{Reg} generation in the thymus when the ligand was expressed by radio-resistant cells of the entire animal (35) or even when agonist ligand-expressing thymic epithelium (23) was transplanted into TCR transgenic mice. When the same ligand was expressed exclusively on hematopoietic cells (mostly B cells), predominantly $CD4^+25^-$ T_{Reg} were generated (23). Additional data showed that naive mature T cells could become both $CD25^-$ and $CD25^+$ T_{Reg} when they were exposed to agonist ligands on peripheral hematopoietic tissue (23). This somewhat bewildering variety of origins and phenotypes of T_{Reg} suggests that the mode of agonist–ligand presentation is of crucial importance in determining whether and if so which phenotype of T_{Reg} is generated. A thorough in vivo analysis of these phenotypically distinct T_{Reg} is required in order to determine whether some of these correspond to a distinct lineage of T_{Reg} cells in addition to T helper (T_H) cells and killer T cells and/or whether some of these cells correspond to effector cells, much like so-called T_{H1} or T_{H2} cells, that apparently require continuous exposure to Ag to be maintained in a particular state of differentiation.

It is probably reasonable to focus initially on $CD25^+$ T_{Reg} cells generated intrathymically, since such cells are likely to have an important role in tolerance to self. However, T_{Reg} that can be generated through antigenic exposure of naive $CD4^+$ T cells are of obvious interest for therapeutic interventions. The latter type of cells is probably similar to that previously generated either in vitro (36) or after transfer of naive T cells into allogeneic hosts (37).

A Lineage of T_{Reg} Cells? With information of how some T_{Reg} cells are generated in vivo, one can now proceed and ask whether Ag recognition is only required for the induction of T_{Reg} in the thymus or whether it is in fact also necessary to maintain cells with that phenotype in peripheral lymphoid tissue. This can best be addressed by transfer of T_{Reg} cells with a transgenic receptor from $RAG^{-/-}$ TCR transgenic mice also expressing the relevant Ag into recipients lacking the relevant Ag. In one recent report, it could be shown that such T_{Reg} cells could persist for several weeks without cell division, maintaining their cell surface phenotype and the ability to suppress proliferation of naive T cells in vitro (1). This agrees well with the finding that some $CD25^+$ T_{Reg} from normal mice that express high levels of CD62L can survive as quiescent cells for >70 d without changing their phenotype (2). These data strongly argue that at least some of the $CD25^+$ T_{Reg} cells belong to a lineage with a "suppressor program" that remains stable over long periods of time rather than representing Ag-dependent short-lived effector cells. In the same transfer experiments, some recently activated T_{Reg} do not survive nearly as well (2), and it needs to be established whether this is because of prior prolonged exposure to Ag.

How Anergic Are Different T_{Reg} Cells In Vivo? Somewhat unexpected from the in vitro data, several groups observed independently (1–4) that in normal nonlymphopenic mice T_{Reg} cells can be stimulated by Ag to proliferate almost as strongly as naive $CD4^+$ T cells such that a marked accumulation of T_{Reg} can be observed in Ag draining LNs. During the proliferative phase, T_{Reg} cells upregulate CD25 expression even further. After 8 d of continuous expansion, such cells are still anergic in the in vitro assay and able to suppress the proliferation of naive T cells. What is the reason for this discordant in vitro and in vivo behavior? Two obvious possibilities exist. First, there may be more IL-2 available in vivo allowing for better proliferation. This would be consistent with the notion that high doses of IL-2 were permissive for T_{Reg} proliferation in vitro. On the other hand, in the standard culture conditions DCs were absent, and presentation of Ag by those cells in vivo may be key to inducing proliferation. These possibilities were to some extent addressed by one of the reports (3): stimulation of cocultures of T_{Reg} on naive T cells with activated DCs resulted in proliferation of naive cells, presumably because such culture conditions make naive T cells insensitive to suppression by T_{Reg} (38). This could represent an important observation that may explain how naive T cells can escape suppression when presented with Ag on activated DCs. However, although this has been described in vitro (3, 38), the in vivo relevance of the observation is not clear as in vivo responses of naive T cells to Ag presented by fully activated DCs were strongly suppressed by regulatory T cells (39). Interestingly, under the in vitro conditions, not only naive T cells but also T_{Reg} proliferated perhaps in an IL-2–dependent manner, since the proliferation could be (incompletely) inhibited by IL-2 receptor antibodies in spite of the fact that IL-2 production by the T_{Reg} cells was below the level of detection (36). In vitro stimulation by mature DCs may be required for some minimal IL-2 production by T_{Reg}, whereas in vivo the combined action of Ag presentation by DCs and the availability of exogenous IL-2 may help T_{Reg} expansion. Here it should be emphasized that even in vivo T_{Reg} produce little

IL-2 themselves (1). Thus, at least some T_{Reg} are capable of marked Ag-induced expansion in vivo in spite of their anergy in vitro. This does not necessarily apply to all T_{Reg}, especially those that are constantly stimulated by Ag in vivo such as $CD25^+$ and $CD25^-$ T cells from mice that express Ag on hemopoietic cells. The proliferation of such cells could not be efficiently rescued by IL-2 in vitro, and they did not expand when coinjected with Ag-pulsed DCs in vivo (23). On the other hand, the potential of not recently activated T_{Reg} to expand in vivo seems somewhat limited: it was noted that when T_{Reg} were injected into mice that expressed the relevant Ag under control of the insulin promoter they initially expanded significantly in pancreatic LNs but eventually disappeared by day 11, perhaps due to cell death (2). Curiously, in a different model T_{Reg} did not disappear up to day 14 (4).

The important question whether or not Ag-induced proliferation of T_{Reg} in vivo requires activated DCs was likewise addressed in various ways. Recent reports agree that there is no necessity for activation of DCs since intravenous injection of soluble protein or presentation by tissue such as pancreatic islet cells was sufficient to induce proliferation, which in the latter case was more limited and occurred presumably after uptake of Ag by DCs (1–4). This suggests that Ag presentation by immature DCs does not only induce deletion of naive T cells after a short proliferative wave (10, 11, 39, 40) but can induce marked expansion of T_{Reg} cells which, however, eventually may disappear when Ag is continuously present (2).

Does Expansion of T_{Reg} In Vivo Require IL-2? The apparent IL-2 dependence of T_{Reg} expansion in vitro could suggest that this cytokine plays also an essential role of T_{Reg} expansion in vivo, and recent data in IL-2 and IL-2R–deficient mice are consistent with that notion (41). IL-2R β chain knockout mice suffer from autoimmunity that can be cured if the IL-2R β chain is expressed as a transgene in such mice in the thymus only. Such IL-2Rβ transgenic IL-$2R\beta^{-/-}$ mice when compared with IL-$2R\beta^{-/-}$ mice have more $CD4^+25^+$ cells in both the thymus and secondary lymph organs presumably because of increased emigration from the thymus. Autoimmunity in IL-$2R\beta^{-/-}$ mice can also be cured by transfer of $CD4^+25^+$ cells from normal mice which expand in the host (41, 42). However, transfer of IL-2Rβ transgenic peripheral $CD4^+25^+$ cells from IL-$2R\beta^{-/-}$ mice, which no longer express the IL-2Rβ chain, into IL-$2R\beta^{-/-}$ recipients does not cure disease, and these cells do not expand presumably because they have lost IL-2R transgene expression and thus cannot utilize IL-2. Although it is not clear to what extent the expansion of T_{Reg} is Ag driven in these particular experiments, these data raise the possibility that Ag-induced in vivo expansion of T_{Reg} likewise requires IL-2. In addition, IL-2 may have an essential role in the generation of T_{Reg} in the thymus, since IL-$2^{-/-}$ mice were reported to have no $CD4^+25^+$ cells in the thymus (42). It is of interest that even in the presence of IL-2 the expansion of T_{Reg} is limited, perhaps by a homeostatic mechanism that prevents suppression of the entire immune system (42).

In Vivo Suppression and Cytokine Production by T_{Reg}. Analogous to the in vitro coculture experiments, one can now begin to analyze in which way T_{Reg} interfere with the generation of T effector cells by coinjecting T_{Reg} with naive T cells with known Ag specificity into normal mice and following their proliferation and differentiation (1–4). When this was done with an initially low frequency of both T_{Reg} and naive T cells representing each 0.3% of $CD4^+$ T cells, both populations expanded in an Ag-dependent manner and produced typical patterns of cytokines, the T_{Reg} mostly IL-10 and the naive T cells IL-2 and γ-interferon. At later points in time, the accumulation of effector cells derived from naive T cells was severely hampered by the expanding T_{Reg} cells such that by day 8 after antigenic stimulation the latter represented the dominant population. The diminished numbers of descendants of naive T cells when briefly stimulated with PMA and ionomycin in vitro, however, exhibited no significant changes in cytokine content, neither IL-2 nor IFN-γ (1). Thus, contrary to in vitro studies, no influence of T_{Reg} on commitment of T cells to cytokine secretion, i.e., immunomodulation, could be observed. If such immunomodulated cells were to exist in vivo, they may have been quickly removed by cell death or migrated from the Ag draining LN even though no significant changes were observed in nondraining nodes. Thus on the basis of these data, a reevaluation of the effects of T_{Reg} activity on naive T cells seems warranted. The initial lag phase in the inhibition of naive T cell expansion may be related to the fact that in vivo, not unlike in vitro, close proximity of T_{Reg} and their targets is required also and that both populations must reach a certain frequency before their effective interaction. The secretion of IL-10 may help immunosuppression under certain but not other conditions and other forms of suppression, that may include competition for growth factors, may represent the most effective way in which regulatory T cells interfere with immunity (1).

Defects in Recessive or Dominant Tolerance in IL-$2^{-/-}$ and IL-$2R^{-/-}$ Mice? The fact that T_{Reg} expansion is required for effective immunosuppression and that the expansion may require IL-2 raises the question whether the autoimmunity in IL-$2^{-/-}$ or IL-$2R^{-/-}$ mice is due to failure of recessive or dominant tolerance. Initially, it was suggested that IL-2 was necessary to make activated T cells susceptible to Fas-dependent apoptosis (43–45), and thus it was considered that defects in activation-induced cell death were responsible for autoimmunity in IL-$2^{-/-}$ or IL-$2R^{-/-}$ mice. It has been realized, however, that the proapoptotic member of the Bcl2 family, Bim, rather than Fas is essential for activation-induced cell death in vivo (46, 47), whereas Fas appears to have a major role in apoptosis in vitro. Thus, there is ample reason to suspect that defects in dominant rather than recessive tolerance are responsible in mice that cannot utilize IL-2 because of genetic defects. As discussed above, this scenario is strongly supported by the fact that $CD4^+25^+$ regulatory T cells with intact but not deficient IL-2 receptors (IL$R\beta^{-/-}$) can cure the autoimmune disease in IL-$2R\beta^{-/-}$ mice (41). Thus, these data warrant reexamination of conclusions on the mechanisms of autoimmunity

in various gene-deficient mice, including Fas-deficient mice, since it is in fact not even clear whether the lymphadenopathy and autoantibody production in Fas-deficient mice is due to defects in Fas-dependent apoptosis in T cells (48).

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