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PERSPECTIVE

Natural regulatory T cells and self-tolerance

Ronald H Schwartz

The adaptive immune system allows individual organisms to mount defensive reactions against unanticipated pathogens by developmentally creating a diverse repertoire of clonally distributed receptors capable of recognizing a multitude of antigens and then expanding as effector cell populations those that can recognize molecules from the pathogens. To function properly, the system must deal with the problem of randomly generated receptors that can recognize self components. Most solutions to this self-tolerance problem are cell intrinsic and involve the deletion or inactivation of autoreactive cells. However, an extrinsic form of dominant tolerance has been demonstrated that takes the form of CD4$^+$ regulatory T cells. This perspective discusses why such a mechanism might have evolved and the problems it presents for self–non-self discrimination.

In 1905 Ehrlich and Morgenroth published the first experiments to suggest that individuals could not be easily immunized against their own tissues[1]. Goats could make an antibody response against the blood cells of other goats but not against their own blood cells. Owen subsequently suggested that this tolerance of self tissues is acquired during the development of the immune system, based on observations that dizygotic bovine twins, in contrast to normal cattle, were incapable of making anti–blood cell responses against each other's cells because of hemopoietic cell chimerism that developed through blood vessel anastomoses in their fused placentas[2]. In 1953, Billingham, Brent and Medawar carried out the first lab experiments to explore the cellular basis of this immunological tolerance[3]. In their Nobel Prize–winning work, they injected allogeneic tissues into fetal mice *in utero* and found that after the animals reached maturity, they were greatly impaired in their ability to reject skin grafts from the same allogeneic mouse strain but not a third-party graft from a different allogeneic mouse strain. This rejection deficiency could be corrected if the tolerant mice were given primed lymph node cell populations. The mechanism proposed by Burnet for this acquired tolerance process was selective clonal deletion of the lymphocytes specific for the alloantigens injected during development[4]. However, experiments attempting to reconstitute the tolerant animals with normal lymph node populations were much less effective, suggesting that things might be a bit more complicated[5]. Nonetheless, subsequent work with T cell receptor (TCR)–transgenic mice[6] and mice expressing mouse mammary tumor virus[7] demonstrated that self-reactive T lymphocytes can be clonally deleted in the thymus by an apoptotic process[8].

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Non–clonal deletion mechanisms

Not all of the observed tolerance phenomena, however, can be easily explained by a clonal deletion model. In the case of B lymphocytes, autoreactive cells can remain alive after the negative selection process. Some of the cells seem to have substituted one of their surface immunoglobulin chains, in a process referred to as 'receptor editing', which reduces their specificity for the self antigen[9,10]. Others seem to escape the bone marrow with the same receptor, but they do not seem to respond to cognate antigen very well, a process referred to as 'clonal anergy'[11]. Experiments in the T cell compartment have also suggested that receptor editing[12] and anergy[13,14] might be going on in the thymus. In the presence of the antigen, TCRα chains have been replaced and unresponsive peripheral T cells with the same transgenic TCR have been found. However, a unique self-tolerance mechanism attributed to the thymus is the generation of antigen-specific regulatory T cells (natural T_{reg} cells). The earliest experiments to suggest the existence of such cells were by Nishizuka and Sakakura[15], who showed that mice thymectomized between 2 and 4 days of age develop organ-specific autoimmune disease, which could be prevented by 'adding back' syngeneic T cells obtained from adult thymus or spleen. Sakaguchi subsequently characterized these negative regulatory cells as the CD4$^+$CD25$^+$ natural T_{reg} cells that express Foxp3 (refs. 16,17). In addition, LeDouarin and colleagues showed that grafts of thymic epithelial anlage are required for the induction of acceptance of allogeneic and xenogeneic limb bud grafts[18]. This process was subsequently shown to be a dominant form of tolerance involving CD25$^+$ natural T_{reg} cells[19].

Natural T_{reg} cells as a separate lineage

So is there a separate lineage of CD4$^+$ regulatory T cells involved in self-tolerance that develops in the thymus? The competing hypothesis is that such cells develop in the periphery and circulate back to the thymic medulla after antigen stimulation. As summarized in the reviews by Sakaguchi[20] and Fontenot and Rudensky[21] in this issue, cells can be found in newborn thymus at day 2 with all the markers and functional properties of natural T_{reg} cells, before there are many T cells of any kind detected in the spleen[22]. Furthermore, immature CD4$^-$CD8$^-$ double-negative, Thy-1.2-marked thymocytes injected into the thymuses of irradiated Thy-1.1 mice develop into CD4$^+$CD25$^+$ T cells within 1 week. The demonstration that natural T_{reg} cells express the transcription factor Foxp3 has also been inter-

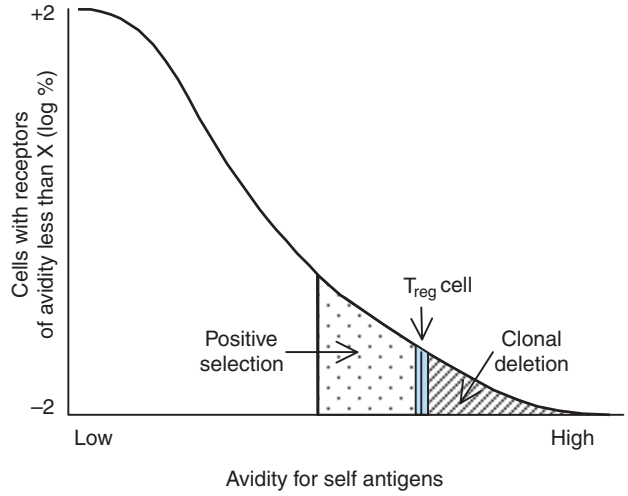

Figure 1 Selection of the T cell repertoire as a consequence of TCR avidity for self antigens expressed in the thymus. Cells whose receptors have an avidity greater than that indicated by the vertical bar (middle) undergo positive selection. Of those, cells with receptors of the highest avidities (striped area, far right) undergo negative selection by clonal deletion. Natural T_{reg} cells are hypothesized to come from the group of cells whose avidities are near the lower end of the negative selection spectrum and the higher end of the remaining positively selected cells (blue area). The vertical line in this area represents the threshold cutoff for clonal deletion.

preted as support for the idea of their separate lineage. The most persuasive evidence comes from experiments in which expression of Foxp3 in $CD25^-CD4^+$ T cells converts them into $CD25^+$ T cells with a suppressive phenotype[17,23]. Foxp3 and some T_{reg} cell surface markers have also been induced in $CD4^+CD8^+$ double-positive thymocytes by stimulation with antibody to TCR and to CD28 (ref. 24). Nevertheless, the ideal experiment of producing functional natural T_{reg} cells in fetal thymic organ cultures should be done to definitively rule out the recirculation argument. The biggest challenge, however, to the separate lineage hypothesis is the claim that $CD25^-CD4^+$ T cells can be converted to $CD25^+$ $Foxp3^+$ T cells in the presence of cytokines such as transforming growth factor-β during antigen stimulation[25]. Most of those studies, however, did not use limiting-dilution conditions and so the possibility that minor contaminating natural T_{reg} cell populations were selectively expanded was not ruled out. Notably, the one convincing case involves a TCR-antigen pair known to be capable of giving rise to natural T_{reg} cells in a transgenic model[26]. Thus, it remains possible that differentiation to natural T_{reg} cells may be achieved outside the thymus in particular circumstances.

The specificity of natural T_{reg} cells

If natural T_{reg} cells are accepted as a separate lineage, what do they recognize? Studies of major histocompatibility complex (MHC)–deficient mice have demonstrated that the generation of natural T_{reg} cells requires MHC class II expression on cortical epithelial cells[27] and it is likely that the receptors are MHC restricted based on the few examples of natural T_{reg} cells for which the antigen specificity is known[28,29]. They also require CD28 ligation for the induction of Foxp3 in the thymus[24]. However, natural T_{reg} cells do not use all αβ TCRs[30]. In fact, most TCR-transgenic mice made monoclonal by crossing onto a recombination-activating gene–deficient background do not give rise to natural T_{reg} cells. Those that do this require the simultaneous introduction of a foreign antigen[28,29]. Furthermore, those TCRs are a unique subset of the ones with the nominal antigen specificity[28]. These data suggest that their repertoire is intended for the recognition of a very discrete set of antigens. One possibility that has been discussed is the subset of tissue-specific antigens that are expressed in thymic medullary epithelial cells[31]. The conundrum of how the immune system deals with tissue-specific antigens goes back to Medawar, who thought there was no problem because the hematopoietic chimerism of dizygotic cattle led to their acceptance of skin grafts[32]. However, subsequent studies by Boyse and colleagues[33], Emory, and McCaullagh and colleagues[34] showed that weak immune responses against tissue-specific antigens do exist. The expression of such antigens in small clusters of thymic medullary epithelial cells provides a means by which a peripheral problem could be solved centrally[31]. Although a few studies have suggested that such expression can lead to T cell deletion as the tolerance mechanism[35], the possibility that the stimulus also generates natural T_{reg} cells is compelling because of the embryo transplant experiments of LeDouarin and colleagues discussed above[18,19].

A dominant self-tolerance mechanism

If the existence of natural T_{reg} cells that are specific for a subset of self-antigens is accepted, the next issue is why such a dominant tolerance mechanism is needed[36]. To try to resolve this, the T cell repertoire generated by a rearranging set of genes and its potential for great diversity must be considered. This will provide a set of receptors of varying avidities for the targeted set of self-antigens, creating a continuous distribution (**Fig. 1**). A clonal deletion mechanism will remove those cells recognizing self antigens with the receptors of highest avidity, but where is the cutoff set? Will the conditions for selection in the thymus be equivalent to the T cell activation conditions in the periphery during an immune response? 'Clonal deletionists' have argued and presented data to support the hypothesis that the activation threshold for deletion events in the thymus is substantially lower than the response threshold for peripheral T cell activation[37–40]. However, a dominant tolerance mechanism could also solve the problem by converting the potentially destructive effector T cells (T_{eff} cells) to negative regulators (natural T_{reg} cells). These would be the T cells with receptors with sufficiently low avidity for the self antigens that they are not usually clonally deleted by the negative selection process, but on the high end of the avidity spectrum of those receptors that are positively selected[28,30,36] (**Fig. 1**). These would potentially be autoreactive in more strongly activating conditions or even slightly autoreactive in all conditions if they escaped clonal deletion[29,30]. If clonal deletion is 'leaky' even in the highest avidity range, then natural T_{reg} cells could also be generated with higher avidities.

It has been argued that such natural T_{reg} cells would be more powerful than a recessive tolerance mechanism, because, through a 'bystander' mechanism of suppression, they could also downregulate the responses of T cells with receptors that recognize other autoantigens in the same tissue, even if that second antigen were not expressed in thymic epithelial cells[36]. This argument seems particularly persuasive given all the experimental evidence for bystander suppression noted with natural T_{reg} cells[41] *in vitro*[42] and *in vivo*[16,43]. However, it contains a 'logical trap'. If T cells specific for other self antigens can be suppressed, then responses to foreign antigens can also be suppressed. To circumvent this, various quantitative arguments have been made. One is that natural T_{reg} cells are anergic and therefore could not expand their populations as well as activated T_{eff} cells[44]. However, *in vivo*, natural T_{reg} cells seem to have mechanisms for readily expanding their populations[45,46], possibly involving interleukin 2 (ref. 47) and/or Toll-like receptor signaling[48]. Another is that the T_{eff} cells will have a

higher avidity for foreign antigens than the natural T_{reg} cells will have for their self antigens. This might allow the former to respond more quickly, before there is enough tissue destruction to bring high doses of tissue-specific self antigens to the draining lymph node. However, a study of the mechanism of suppression by influenza hemagglutinin–specific natural T_{reg} cells might resolve this controversy[49]. This double-transgenic model (TCR and antigen) generates about 50% T cells in both the natural T_{reg} cell and the T_{eff} cell classes and has provided some of the best data so far regarding the thymic development of natural T_{reg} cells[28,46]. Unexpectedly, both *in vivo* and *in vitro* experiments have demonstrated that the suppression in this model is mediated by direct competition for specific antigen and that there is no bystander suppressive effect on either of two other monoclonal T cell populations, even in an F1 host, which would bring the two T cells together on the same antigen-presenting cell. If this is the true evolutionary function of the generation of natural T_{reg} cells (that is, to suppress low-avidity anti-self T_{eff} cells of overlapping specificity that 'sneak through' clonal deletion), then the bystander problem of suppressing responses to foreign antigens disappears.

Bystander effects

But how can all the bystander effects that have been repeatedly found by many investigators[41] be explained? Perhaps there is a nomenclature problem. It is clear that all forms of $CD4^+$ T cells can acquire the ability to make immunoregulatory cytokines such as interleukin 10, transforming growth factor-β and interleukin 4. Such cells were formerly called T helper type 2 and T helper type 3 and were discussed in terms of immune deviation or class regulation. The existence of some other non-cytokine-mediated mechanisms for bystander suppression that involve direct T cell–T cell interactions[42] should not fundamentally change the function of these states derived after antigen exposure, whose purpose is to downregulate and prevent immunopathologic damage[50] or to deviate the immune response toward a certain type of effector function so that it does not damage the particular tissue in which it occurs[51]. Hence, the term 'inducible T_{reg} cell' as it is now used may be somewhat misleading, for clearly $CD25^+$ natural T_{reg} cells can also be induced to participate in bystander-mediated regulation, for example, involving interleukin 10 in the leishmania system[52] described by Belkaid and Rouse[53] in their review in this issue. These cells are largely indistinguishable in their mode of suppression from T_R1 cells derived from $CD4^+CD25^-$ $Foxp3^-$ T cells[54]. In fact, in helicobacter-specific infections, the T_{reg} cells can be either $CD25^+$ natural T_{reg} cells or T_R1 cells[55]. But how do natural T_{reg} cells become recruited to carry out bystander-mediated suppression if the antigens they recognize are only tissue-specific self antigens? For alloreactivity in transplantation models of natural T_{reg} cells, this is easy to understand in terms of cross-reactivity of their receptors, which are MHC restricted[56]. Roughly 1–5% of such T cells would be expected to be cross-activated by any given allogeneic challenge. In the case of leishmania, however, the basis for a cross-reactive activation is less clear, unless of course the parasite has specifically evolved through molecular mimicry to take advantage of the natural T_{reg} population for its own survival. Finally, a substantial number of T cells emerge from the thymus expressing two TCRs[57]. If one receptor is used to be selected into the natural T_{reg} cell lineage, then the other would be free to recognize and respond to a foreign or self antigen. All the TCR-transgeneic mice that do not generate natural T_{reg} cells on a recombination-activating gene–deficient background can generate such cells on a wild-type background, and at least some of these cells can be involved in the immunoregulation of responses through the unselected transgenic receptor[58]. In this context, there is no reason that humans could not use comparable 'tricks' to expand natural T_{reg} cell populations for use in facilitating organ transplantation and treating autoimmune diseases (discussed in the review by Sakaguchi[20] in this issue).

The adaptive immune system is a powerful tool for defending against invading pathogens. However, its complexity has generated the problem of self tolerance, whose handling has required several fail-safe mechanisms. The existence of autoimmune diseases and chronic infections would indicate that this strategy is not always effective. Thus, it is fair for Hedrick[59] to ask whether evolution of the adaptive immune system was in fact really worth it.

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COMPETING INTERESTS STATEMENT

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