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# REGULATORY T CELLS IN AUTOIMMMUNITY*

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■ **Abstract** Clonal deletion of autoreactive T cells in the thymus is not the sole mechanism for the induction of tolerance to self-antigens since partial depletion of peripheral $CD4^+$ T cells from neonatal and adult animals results in the development of organ-specific autoimmunity. Reconstitution of these immunodeficient animals with populations of regulatory $CD4^+$T cells prevents the development of autoimmunity. The lineage of regulatory $CD4^+$ T cells is generated in the thymus and can be distinguished from effector cells by the expression of unique membrane antigens. The target antigens for these suppressor populations and their mechanisms of action remain poorly defined. Depletion of regulatory T cells may be useful in the induction of immunity to weak antigens, such as tumor-specific antigens. Conversely, enhancement of regulatory T cell function may be a useful adjunct to the therapy of autoimmune diseases and for prevention of allograft rejection.

## INTRODUCTION

Shortly after the discovery in the late 1960s that T lymphocytes functioned as helper cells for B lymphocytes, RK Gershon (1) proposed that T cells could also act as regulatory cells that could suppress the immune response. The prevailing view in the 1970s–1980s was that suppressor T cells mediated their biologic effects by producing soluble factors that were responsible for their biologic activity. The early volumes of this series contained detailed reviews describing the immunologic properties of these factors. Many of these soluble suppressor molecules were claimed to be antigen-specific and contained I-J determinants encoded by the MHC and/or determinants encoded by Ig genes. Molecular studies in the mid-1980s, however, clearly demonstrated that the genes encoding the T cell receptor were not identical to those encoding Ig and failed to identify an I-J gene within the MHC. As succinctly pointed out by Green & Webb (2), the ''S'' word became the nearest thing to a dirty word in cellular immunology, and its use was considered synonymous with over-interpretation of scanty data and mystical phenomenology.

Tolerance still remains a fundamental concept of modern immunology, but clonal deletion of autoreactive T cells in the thymus cannot be the sole mechanism for the induction of tolerance. Many of the in vivo experiments performed over the past 30 years contain compelling data that supports the existence of suppressor T cells. The discovery of Th1/Th2 cells in the late 1980s prompted most workers in the field to abandon the concept that suppressor T cells were a specialized population; suppression was merely the result of the activity of counter-regulatory cytokines. The purpose of this review is to resurrect the concept that the suppressor T cell is a member of a separate lineage of cells that mediates its down-regulatory functions by a variety of effector mechanisms. I focus on the role of suppressor T cells in the prevention of organ-specific autoimmunity and begin with an overview of the animal models initially described 30 years ago, which formed the foundation for more recent studies that have facilitated the characterization of suppressor T cells.

## DEPLETION OF REGULATORY T CELLS FROM NEONATAL ANIMALS

During the course of studies investigating the role of the thymus in mediating tumor immunity, Nishizuka & Sakakura (3) observed that female mice that were thymectomized (Tx) early in life became infertile secondary to the development of oophoritis. Ovarian atrophy was observed when the thymus was removed from neonatal animals on day 3 of life and not on day 1 or day 7. Thymus grafting at 7 days of age prevented the development of disease, while grafting at 40 days of age had no effect. Later studies demonstrated that grafting of an intact thymus was not required, as disease could be prevented by injection of a suspension of thymocytes from 7-day-old or adult mice or of spleen or lymph node cell suspensions from adult mice. In contrast, grafting of allogeneic thymus, syngeneic newborn spleen, injection of bone marrow cells, spleen cells from 7-day-old intact donors, or spleen cells from adult d3Tx donors failed to protect. The conclusions drawn from these studies were that thymus-derived lymphocytes were responsible for the suppression of disease and that the cells that prevented the disease in adult spleen were derived from the thymus. Although these cells developed in the thymus of the neonate, they were not peripheralized to spleen or lymph nodes during the first 3 days of life since spleen cells of newborn mice were not effective in preventing disease and d3Tx completely extinguished this lineage. These investigators considered the possibility early on that a suppressor factor might be extractable from the thymus, which would be effective in vivo, but crude extracts from the thymus did not substitute for the intact thymus! Thus, they did not embark on the treacherous journey of the ‘‘mainstream’’ immunologists who had simultaneously discovered suppressor T cells.

The syndrome of post-d3Tx-induced organ-specific autoimmunity was characterized in depth over the next 10 years (4, 5). In addition to autoimmune oophoritis and orchitis in A strain mice, thyroiditis was most prominently observed in C3H mice, while autoimmune gastritis was most often seen in BALB/c mice. The gastritis was characterized by a loss of chief and parietal cells and by varying degrees of lymphoid infiltration along thickened gastric muscularis mucosa. Anti-parietal cell antibodies were present in the sera of mice with gastritis and megaloblastic anemia subsequently developed; the gastritis in BALB/c mice resembles autoimmune pernicious anemia in humans. Other organs involved included the coagulating glands, prostate, and epididymus. More than one organ could be involved in a single mouse, but the disease process was completely organ-specific, with no evidence of the development of systemic autoimmune disease, antinuclear antibodies, immune complex nephritis, or manifestations of graft-versus-host disease (GVHD). Only small numbers ($15 \times 10^4$) of regulatory T cells were needed to prevent disease from developing in d3Tx mice, while much larger numbers of cells ($10^7$) were used to demonstrate suppression of antibody formation (1). Autoimmune oophoritis or gastritis was transferred successfully into newborn mice or adult *nu/nu* mice with splenic T cells (Thy-$1^+$, Lyt-$1^+$, Lyt-$23^-$) obtained from d3Tx mice with disease (6, 7); both organ-specific lesions and circulating autoantibodies developed in the recipients. The effector cell was sensitive to antithymocyte serum, but resistant to cyclophosphamide treatment or in vitro X-irradiation. Later studies (8, 9) demonstrated that the effector and the suppressor T cell populations were $CD4^+CD8^-$.

Although the reconstitution experiments strongly implied that disease was prevented by suppressor T cells, a major advance in this field was the demonstration (10) that removal of suppressor cells T cells from an immune system of an otherwise normal animal resulted in disease, and that reconstitution of the recipient with suppressor T cells then reestablished self-tolerance and prevented autoimmunity. When T cells from normal adult mice were separated based on the relative expression of the Lyt-1 (CD5) antigen, transfer of the Lyt-$1^{low}$ cells to *nu/nu* mice resulted in the development of autoimmune diseases in several organs. In contrast, transfer of the Lyt-$1^{high}$ T cells failed to result in autoimmunity and cotransfer of Thy-$1^+$, Lyt-$1^{high}$ cells with Lyt-$1^{low}$ cells completely prevented the development of disease. Potentially pathogenic $CD4^+$ self-reactive T cells were present in the periphery of normal mice, and their activation/expansion was controlled by $CD4^+$ cell suppressor cells. It is ironic that this clear demonstration of the presence of suppressor T cells in normal mice was published at about the same time that the entire field of suppression was in a state of chaos, since molecular cloning of the TCR $\alpha$- and $\beta$-chain genes ruled out the presence of the Ig determinants, which were purported to be associated with many of the soluble T cell factors.

Very little progress was made in the further characterization of the suppressor T cell population involved in this model of organ-specific autoimmunity over the next 13–14 years. One problem was that the Lyt-1 antigen was actually expressed on all T cells and that the suppressor cells were contained in the large subset (80–

90%) of cells susceptible to lysis by anti-Lyt-1 and complement. The next seminal advance in the field was again made by Sakaguchi and associates (11, 12), who further defined the suppressor cell in this model as a minor (10%) subset of $CD4^+$ T cells which coexpressed the CD25 (IL-2R α-chain) antigen. When $CD4^+CD25^-$ T cells from normal BALB/c mice were transferred to 6-week-old syngeneic *nu/nu* recipients, the mice developed inflammatory lesions in various organs that resembled the pattern of autoimmune disease seen post-d3Tx; some recipients also developed pathologic changes consistent with systemic autoimmunity, including polyclonal B cell activation and hypergammaglobulinemia, proteinuria secondary to immune complex glomerulonephritis, and a wasting disease that clinically resembled GVHD. Most importantly, disease could be prevented by cotransfer of $CD25^+$ T cells within 10 days of transfer of the CD25- cells.

In order to demonstrate that a deficiency of the same $CD25^+$ population of regulatory T cells was also responsible for the autoimmunity seen post-d3Tx, d3Tx mice were inoculated on day 10 of life with either whole spleen cells or spleen cells from which $CD25^+$ T cells had been depleted (13). Autoimmune gastritis was abolished only in those recipients that were reconstituted with $CD25^+$ T cells. A logical extension of this finding would be that $CD25^+$ T cells would not be detectable in the peripheral lymphoid tissues of unmanipulated 3-day-old mice. While Asano et al (12) could not detect $CD4^+$ $CD25^+$ in the spleen of 3-day-old mice, this result is difficult to interpret, as $CD3^+$ T cells cannot be detected in the spleen until after day 3 of life. In contrast, Suri-Payer et al (14) could readily identify $CD4^+$ T cells in lymph nodes of mice as young as 2 days of age, and 10% of these $CD4^+$ T cells expressed CD25; it is possible that the $CD25^+$ cells home to the lymph node in the neonate rather than the spleen. The presence of $CD25^+$ T cells in 2-day-old animals raised the question of whether they actually were responsible for preventing autoimmunity. It remains possible that they are nonfunctional or even that they may represent effector cells that have been induced to express CD25 following recognition of their autoantigen. Alternatively, the small number of $CD25^+$ cells present on day 2 of life may not expand in the periphery after d3Tx, and their number may be too low to mediate immunoregulation. Interestingly, d3Tx resulted in an immediate increase in the percentage of $CD4^+CD25^+$ cells, which reached a plateau of approximately 30% of total $CD4^+$ T cells 10 days after Tx. These cells are likely effector cells that have been very rapidly activated after d3Tx.

A number of other protocols for the induction of the lymphopenic state have been described that resulted in the development of a spectrum of autoimmune diseases which closely resembles that seen post-d3Tx; presumably, all of these manipulations result in selective depletion and/or delayed development of the suppressor $CD4^+CD25^+$ populations. When newborn mice were treated with cyclosporine A (CsA) during the first 7 days of life or when adult mice were treated for 2 weeks, transplantation of their thymuses to *nu/nu* recipients resulted in the development of organ-specific autoimmunity (15, 16). Cotransplantation of normal thymus with CsA thymus or injection of spleen cells from normal adult

mice prevented autoimmune disease. d7Tx of CsA-treated newborn mice markedly enhanced the development of disease; thymocytes from hydocortisone-treated mice contained functionally active regulatory T cell populations (17). Administration of CsA to euthymic newborn, but not adult, mice caused organ-specific autoimmune diseases. Although CsA can selectively deplete regulatory T cells from the thymus, autoimmune disease will only develop when regulatory T cells are absent from or have not yet migrated to the periphery.

High-dose (42.5 Gy) fractionated (2.5 Gy, 17 times) total lymphoid irradiation (TLI) of mice also resulted in the development of organ-specific autoimmunity (18). Radiation-induced tissue damage was not the primary cause of the autoimmune disease because irradiation of the target organs alone failed to elicit autoimmunity and shielding the organs from irradiation was unable to prevent it. Inoculation of spleen, thymocyte, or bone marrow cell suspensions within 2 weeks of TLI prevented the development of autoimmunity. In contrast to the experiments of Penhale et al (19) in the rat model (vide infra), TLI alone was more effective than Tx + TLI in inducing autoimmunity. In the mouse model, the T cells regenerating from the irradiated thymus may be selectively enriched in autoreactive effectors, which may mediate the autoimmune responses synergistically with those derived from the peripheral T cell pool. Neonatal infection with mouse T lymphotropic virus (MTLV) resulted in depletion of $CD4^+$ T cells from the thymus and periphery and the subsequent development of organ-specific autoimmunity (20). Reconstitution of the infected animals 3 weeks after neonatal virus infection with syngeneic adult $CD4^+CD25^+$ T cells from noninfected mice prevented the autoimmune disease. MTLV infection of the newborn appears to selectively impair suppressor T cell function.

All of the models described above for induction of autoimmunity likely result from depletion of regulatory T cells from the thymus and/or the peripheral lymphoid tissue. One of the most intriguing models for induction of autoimmunity described by Sakaguchi and associates (21) appears to involve a delayed maturation of the regulatory population. Transgenic mice that expressed any rearranged TCR α-chain transgene, but not TCR β-chain gene, under control of the IgH chain enhancer developed T cell–mediated autoimmune disease in multiple organs in a single mouse. The disease could be transferred by T cells that expressed endogenous TCR α- and β-chains. It is not known in this model how transgene expression resulted in the induction of autoreactive effector T cells. Although it is theoretically possible that expression of the transgene leads to the production of more self-reactive cells, which then overwhelmed the regulatory cells, it is more likely that transgene expression selectively inhibited or delayed the development of the suppressor population. I favor this second possibility as we (A Thornton, EM Shevach, unpublished observations) have been able to isolate $CD4^+CD25^+$ T cells from adult TCR α-chain alone transgenic mice, which are potent inhibitors of T cell activation in vitro. Thus, expression of the transgene may create a window of opportunity (a transient 3dTx effect) for the self-reactive

T cells that emerge from the thymus to initiate autoimmune damage in the absence of regulatory T cells in the periphery.

For many years one of the ''standard'' protocols for the selective depletion of suppressor T cell function in vivo involved the treatment of animals with cyclophosphamide. Studies by Barrett et al (22) offer some support for this procedure. Autoimmune gastritis was induced in adult BALB/c mice by Tx at 6–8 weeks of age followed by a single dose of cyclophosphamide (300 mg/kg). This treatment transiently reduced the number of splenic T and B cells 25-fold, but by day 8 after treatment, the number of splenic T and B cells had returned to normal adult levels. Cyclophosphamide without Tx did not have any effect. The autoimmune gastritis that developed was identical to that seen post-3dTx, and it could be transferred from gastritic mice to *nu/nu* recipients. A more careful analysis of the susceptibility of the $CD4^+CD25^+$ population to depletion by cyclophosphamide is clearly warrented.

The autoimmune gastritis that develops post-d3Tx is one of the few animal models where the target antigen has been clearly defined. Circulating autoantibodies in post-d3Tx gastritis and in pernicious anemia in humans specifically react with the 95-kDa α-subunit and the 60–90 kDa β-subunit of the membrane bound proton pump, the H/K ATPase (23). To define the target antigen for $CD4^+$ T cells in d3Tx induced gastritis, Alderuccio et al (24) produced transgenic mice that expressed the β-subunit of the H/K ATPase under control of an MHC class II promoter with resultant widespread tissue expression of the β-chain. Transgenic expression prevented the production of autoantibodies and the development of gastritis following d3Tx. The incidence of oophoritis in the transgenic mice was the same as that seen in control mice. Since the transgene was expressed in the thymus, it was likely that tolerance induction occurred in the thymus. Indeed, thymocytes from normal adult BALB/c mice, but not the β-subunit transgenics, transferred autoimmune gastritis to nu/nu recipients. mRNA for the α-subunit gene was easily detectable in normal thymus (25) and transgenic expression of the α-subunit in the thymus failed to prevent the development of autoimmune gastritis. It was postulated that high-affinity T cells specific for the β-subunit could escape to the periphery and be capable of initiating autoimmune gastritis in the absence of regulatory T cells. In contrast, high-affinity T cells specific for the α-subunit would be deleted in the thymus, whereas the low-affinity cells escape to the periphery. These low-affinity cells would not be activated following d3Tx but might be recruited by determinant spreading following the inflammatory response initiated by the activation of β-subunit reactive T cells by Tx; responses to the α-subunit may represent secondary events.

Suri-Payer et al (26) demonstrated that H/K ATPase-reactive $CD4^+$ T cells could be identified in d3Tx mice with gastritis. The frequency of ATPase-reactive T cells was highest in the gastric lymph node since proliferative responses of lymphocytes isolated from more distal sites gave undetectable responses. More detailed analysis (14) of the fine specificity of the $CD4^+$ H/K ATPase-reactive cells demonstrated that freshly explanted gastric lymph node cells from the d3Tx

mice were highly reactive to the α-subunit, but only minimally reactive to the β-subunit of the H/K ATPase. Two T cell clones were isolated that recognized distinct peptides of the α-subunit. Although one clone secreted a Th1 pattern of cytokines while the other demonstrated a Th2 cytokine profile, both clones were equally potent in inducing gastritis with distinct profiles of cellular infiltration in *nu/nu* recipients. The capacity of either of the cell lines to induce disease was abrogated by cotransfer of $CD4^+CD25^+$ T cells from normal BALB/c mice. It is unlikely that the target epitopes recognized by these two clones were immunodominant for the pathogenesis of gastritis because proliferative responses to these peptides were not observed in freshly explanted gastric lymph node cells from animals with gastritis. The preferential reactivity of the $CD4^+$ T cells in proliferation assays with the α-subunit should be contrasted with the studies in the H/K ATPase transgenic mice, where reactivity to the β-subunit played a key role in induction of disease. It remains possible the α-chain dominant proliferative responses were observed at a time when determinant spreading had already occurred or that more complex mechanisms are operative in the protection of the β-, but not α-, subunit transgenic mice from disease post-d3Tx.

One of the most intriguing questions that remains to be addressed in the immunopathogenesis of autoimmunity post-d3Tx is why only certain organs are targeted for involvement by the autoimmune process. When transgenic mice, which expressed the β-subunit of the H/K ATPase in the pancreatic islets under the regulation of the rat insulin promoter, were subject to d3Tx, they developed both gastritis and insulitis (27). The peri-insulitis, however, did not progress to invasion of the islets or to diabetes. Insulitis was only observed in animals that developed gastritis. It was concluded from this study that tissue-specific factors must play a fundamental role in the development of organ-specific autoimmunity. One possibility is that the high turnover of gastric parietal cells (23-day half-life) generated a higher level of antigen presentation in the stomach than in the pancreas. Antigen-specific T cell activation would first occur in the stomach, and then the activated T cells would infiltrate the pancreas. The lack of islet cell destruction may be due to the inability of the infiltrating cells to be restimulated, lack of appropriate cytokines, or the inability to recruit $CD8^+$ T cells.

## DEPLETION OF REGULATORY T CELLS FROM ADULT ANIMALS

The theory that regulatory T cells control antibody production was rapidly applied to the study of autoimmunity by Penhale and colleagues (19, 28, 29), who hypothesized that antibody mediated autoimmune diseases might develop because of a failure of regulatory T cells to control autoantibody production. In a series of innovative experiments, procedures were devised to deplete the regulatory T cells and leave the helper T cell population responsible for autoantibody production

intact. The disease model selected for study was autoimmune thyroiditis because circulating antibody to thyroglobulin was believed to play an important pathogenic role. Spontaneous thyroiditis developed in 60% of Wistar rats following the selective depletion of T cells by Tx and irradiation. Tx was performed between 3 and 5 weeks of age, and the rats were given 4–5 repeated doses of 200 rad at 14-day intervals. Circulating IgG antibody to thyroglobulin also developed, and it was assumed that the major effector cell in this model was the B cell that produced the autoantibody; no other manifestations of autoimmunity were seen. No evidence of thyroiditis was seen in rats that received only local irradiation to the thyroid region, indicating that irradiation itself did not induce pathologic changes.

The conclusion drawn from these studies was that in the unmanipulated animal, B cells autoreactive with thyroid antigens were prohibited from differentiating into autoantibody producing cells by an active controlling T cell mechanism. Although not specifically tested at the time, it was assumed that the suppressor T cell population was mediating its functions by acting directly on the B cell and not by regulating other T cells. The active role of T cells in preventing the development of autoimmunity in this model was confirmed by reconstituting the Tx-irradiated mice with lymphoid cells from normal donors (29). Lymph node, spleen cells, or thymocytes would abrogate disease when administered intravenously shortly after the final dose of irradiation. A limited characterization of the suppressor cells with the reagents available at that the time suggested that they were T cells; they also appeared to have been activated in vivo, as they were found in the fractions containing large cells when separated on a Ficoll gradient. Taken together, these experiments demonstrated that normally autoreactive helper and suppressor cells may coexist and that certain autoimmune responses are held in check by the equilibrium favoring suppressor activity. Although Penhale and colleagues (30) also demonstrated that autoimmune diabetes would develop in a strain of rats that was normally not susceptible to this disease by the Tx-irradiation protocol, this potentially powerful experimental model for the characterization of regulatory T cells in autoimmunity was largely ignored by other investigators for the next 15 years.

Powrie & Mason (31) were the first to use a combination of cell surface markers and functional differences between T cell subsets to define regulatory and effector T cell populations. In the rat, $CD4^+$ T cells could be divided into two subsets based on their differential expression of the CD45RB isoform. $CD45RB^{high}$ T cells mediated GVHD and produced IL-2 and IFN-γ, but little IL-4, upon polyclonal activation in vitro. In contrast, the $CD45RB^{low}$ cells provided the majority of help for secondary antibody production both in vivo and in vitro and produced significant amounts of IL-4, but less IL-2 and IFN-γ. Most importantly, when athymic rats were reconstituted with small numbers of $CD45RB^{high}$ T cells, they developed a severe wasting disease characterized by extensive mononuclear cell infiltration in the lungs, liver, thyroid, stomach, and pancreas 6–10 weeks later. No pathology developed in animals that received unseparated $CD4^+$

T cells or CD45RB$^{low}$ cells. It seemed likely from these studies that the CD45RB$^{low}$ subset controlled the capacity of the CD45RB$^{high}$ subset to mediate the wasting disease. The suppressive effects of the CD45RB$^{low}$ subset were directly demonstrated by Fowell & Mason (32) in the Tx-irradiation model described by Penhale's group (19). Transfer of $5 \times 10^6$ CD45RB$^{low}$ CD4$^+$ T cells completely inhibited the development of diabetes and insulitis. CD45RB$^{low}$ T cells from long-term Tx donors could protect as efficiently as cells from normal donors, demonstrating that the regulatory T cell is long-lived in the periphery.

When mouse T cells were separated on the basis of the differential expression of CD45RB isoforms and injected into immunodeficient SCID recipients, severe autoimmune manifestations were also observed (33–35). In contrast to the rat model in which widely dispersed pathologic lesions were observed, mice that received the CD45RB$^{high}$ subset only developed severe intestinal pathology. Mice that received CD45RB$^{low}$, unseparated CD4$^+$ cells, or a mixture of CD45RB$^{high}$ and CD45RB$^{low}$ cells never developed intestinal lesions. The pathologic changes in the colon included extensive mononuclear cell infiltrates, ulceration, and pronounced epithelial cell hyperplasia. Although the early studies in the rat model suggested that the CD45RB$^{high}$ population was enriched in IFN-γ producers and may represent Th1 cells, while the CD45RB$^{low}$ populations contained IL-4 producers consistent with Th2 cell function, subsequent studies in the mouse model were inconsistent with this functional separation. Indeed, it appeared that, in the normal mouse, the CD45RB$^{low}$ population was enriched in memory T cells, which were primed to be either IL-4 or IFN-γ producers, while the CD45RB$^{high}$ subset contained the majority of naive T cells that had not yet differentiated to produce IFN-γ or IL-4. Not surprisingly, when disease was induced in SCID mice by transfer of the CD45RB$^{high}$ cells, the majority of the CD4$^+$ T cells in the sick animals were found to be CD45RB$^{low}$.

The role of cytokines in disease pathogenesis and the potential contribution of cytokines in protection from disease has been addressed by Powrie and colleagues (36, 37). The presence of colitis in SCID recipients of CD45RB$^{high}$ cells correlated with elevations of IFN-γ, but not IL-4 or IL-10, mRNA in the involved tissues. CD4$^+$ T cells purified from colonic lesions produced 10 times more IFN-γ than did CD4$^+$ T cells from normal BALB/c colons. Treatment of animals with anti-IFN-γ on days 1 and 14 after T cell transfer completely protected animals from disease, and protection was not dependent on the continuous presence of the antibody. Anti-IFN-γ may have permanently altered a step in effector cell differentiation, possibly by inhibiting IL-12R expression (38). In contrast, weekly anti-TNF treatment was required for suppression of disease; when the anti-TNF treatment was stopped at 8 weeks, severe disease was observed in mice sacrificed at 12 weeks. Mice treated with IL-10 were highly protected, but IL-10 also did not cause a long lasting modulation of the immune response, as mice sacrificed 4 weeks after the last treatment with IL-10 developed severe colitis. IL-4 treatment did not affect the induction of colitis, and none of the treatments was associated with induction of Th2 responses.

The approach used (37) to analyze the contribution of cytokines to the protective effects of the CD45RB$^{low}$ cells involved treatment of SCID mice that had been reconstituted with a mixture of CD45RB45$^{high}$ and CD45RB$^{low}$ cells with anti-TGFβ or anti-IL-10R (F Powrie, personnal communication) at the time of reconstitution, and then weekly for 6 weeks. Protection was abrogated in mice treated with either anti-TGFβ or anti-IL-10R when the mice were killed on week 7. There was no evidence that IL-4 played any role in mediating protection since CD45RB$^{low}$ T cells from IL-4-deficient mice were able to inhibit colitis and wasting disease. The regulatory T cell population differs from classical Th2 cells, which either would produce IL-4 or are dependent on IL-4 for their development and differentiation. A similar approach was used in the rat Tx-irrradiation model of autoimmune thyroiditis (39). Both CD45RB$^{low}$ peripheral T cells as well as thymocytes will protect against disease when given immediately following the course of irradiation. When rats were reconsituted with either $10^6$ CD4$^+$ thymocytes or $5 \times 10^6$ CD45RB$^{low}$ peripheral T cells and then treated with either anti-IL-4 or anti-TGFβ for 1 month after reconstitution, protection was abrogated. The potential contribution of IL-10 was not evaluated. Surprisingly, while abrogating suppression, blockade of IL-4 or TGFβ did not alter the basic disease process.

Collectively, these studies suggest that the regulatory T cells are an unusual T cell subset, perhaps related to Th2 or what has been termed Tr1 (40) cells. It should be noted that in both of these models, treatment is continued for many weeks, and the effects of the anti-cytokine reagents may be mediated on cells that are induced to produce these suppressor cytokines and not on the regulatory cells themselves. It is even possible that these cytokines are primarily required for differentiation of the regulatory cell populations in vivo and not for their suppressor effector functions. The requirement for IL-10 in mediating protection in the inflammatory bowel disease model may be indicative of the normal physiologic role of this cytokine in preventing inflammation in the gut since the induction of IL-12 production by bacteria may constantly stimulate Th1 responses to gut antigens. Although these recent studies suggest that certain classes of regulatory T cells mediate their protective effects by producing classic suppressor cytokines, it should also be emphasized that neither of these studies included control groups in which the animals reconstituted with only the CD45RB$^{high}$ subset, which develop disease, were also treated with anticytokine antibodies. It is quite conceivable that both the magnitude and pathology of the disease state in such animals would also have been altered, rendering a comparison with animals in which suppressor function had been abrogated problematic.

## ANTIGENIC SPECIFICITY OF REGULATORY T CELLS

Any approach to an analysis of the mechanism whereby regulatory T cells inhibit organ-specific autoimmunity requires an understanding of their target antigen. Very little progress has been made in this area because suppressor T cells in these

models have not been propagated long term in vitro or cloned. On the other hand, there exists a body of controversial data which suggests that the suppressor populations recognize a target antigen that is specific for the organ under attack. Spleen cells from normal adult male mice were much more effective suppressors of autoimmune orchitis post-3dTx than were spleen cells from female mice or male mice that had undergone a neonatal orchiectomy (41). Spleen cells from female mice were as effective as spleen cells from male mice in preventing gastritis. Studies in prostatitis suggested that the differences were relative rather than absolute, as protection could be seen with $4 \times 10^6$ spleen cells from normal males, but not females, while $4 \times 10^7$ spleen cells from females were protective. Smith et al (8) demonstrated that Con A–stimulated spleen cells from d3Tx male mice were less efficient at transferring oophoritis than were spleen cells from female mice (42). This result suggested that for T cells to initiate oophoritis they may need to be primed by endogenous ovarian antigen. In contrast to the earlier studies (41), normal male, normal female, and spleen cells from females that were oophorectomized at birth were found to suppress d3Tx oophoritis with comparable efficiency. Although these investigators used a wide range of spleen cells ($5–20 \times 10^6$) per recipent, the magnitude of the suppressive effect at the lower doses used was only modest (50–60% suppression with $5 \times 10^6$ cells), and differences between male and female suppressor populations may have been missed.

Taguchi et al (43) used a rather novel approach to study the tissue specificity of the regulatory T cells. The suppressor population that inhibited autoimmune prostatitis again appeared to be organ-specific since $4 \times 10^6$ spleen cells from males, but not females or orchiectomized males, inhibited disease post-d3Tx. All of these populations were equally effective in inhibiting the lacrimal gland adenitis, which also developed post-d3Tx. d3Tx mice, orchiectomized at birth, never developed prostatitis. Prostatitis developed following treatment with dihydrotestosterone, indicating that expression of the prostate antigen responsible for evoking autoimmunity could be induced by the appropriate hormonal stimulus. Most importantly, mice orchiectomized at birth, thymectomized as adults, and then treated with dihydrotestosterone developed a mature prostate. Spleen cells from these mice when inoculated into d3Tx male mice prevented the development of post-d3Tx autoimmune prostatitis. This result demonstrates that prostate-specific regulatory T cells can be activated by the induction of the organ-specific antigen. Activation of these regulatory cells takes place in the periphery since the cells could be isolated from adult Tx hormone-treated mice. It is difficult to reconcile these disparate findings on the presence of tissue-specific suppressors. All the experiments were performed in different disease models involving autoimmune damage to reproductive organs; it is possible that some tissue-specific antigens are not gender-specific, and this may account for the differences between the results with orchitis and oophoritis. Future studies should employ purified populations of $CD4^+CD25^+$ cells rather than unseparated spleen cells and should include very careful dose-response studies.

The term subtractive was initially used by McCullagh (44) to categorize protocols in which the control of the development of tolerance is investigated by preventing the developing immune system from gaining access to an antigen or to the developing organ until self/non-self discrimination had been established. When the fetal rat thyroid gland was destroyed by exposure to radioactive iodine and syngeneic thyroid tissue was implanted into the athyroid rats as adults, autoimmune thyroiditis developed in the grafted tissue. The autoimmune attack was organ-specific, as other endocrine organs did not exhibit signs of inflammation. The development of thyroiditis in rats that had been exposed to radioactive iodine in utero could be prevented by parabiosis to normal syngeneic partners (44). Parabiosis was only protective if instituted at the time of thyroid grafting, but not 1–2 weeks after graft implantation. Normal rats possess migratory regulatory cells capable of blocking the antithyroid immune response of rats in which thyroid development had been disrupted before the development of immunocompetence. These studies on ''subtraction'' of regulatory T cells by removal of the target organ during fetal life are quite consistent with the results described earlier on the failure to detect regulatory T cells in animals in which the reproductive organs were extirpated in the neonatal period. A critical time period of exposure is therefore needed to allow for the development of organ-specific suppressor T cells. Seddon & Mason (45) used the approach developed by McCullagh (44) to demonstrate that peripheral $CD45RB^{low}$ T cells from rats rendered athyroid were unable to prevent thyroid-specific autoimmunity induced by the adult Tx-irradiation protocol. The loss of regulatory cells was specific for the extirpated organ, as T cells from the athyroid rats could prevent the development of diabetes. In contrast, $CD4^+CD8^-$ thymocytes from the same athyroid donors were as effective as those from normal rats at preventing thyroiditis. Regulatory T cells were generated normally in the thymus in the absence of their target organ, but the target organ was needed for survival and/or expansion.

## IN VITRO MODELS OF SUPPRESSOR T CELL FUNCTION

There is little doubt that the immunoregulatory T cells described in this review have potent inhibitory functions in vivo. As the model systems described frequently require weeks to months of assessment of disease activity, it has been difficult to determine the mechanism of action, antigen specificity, or cellular targets of the suppressor T cell populations. A number of recent studies (46, 47) have demonstrated that $CD4^+CD25^+$ T cells are potent inhibitors of polyclonal T cell activation in vitro. Purified $CD4^+CD25^+$ were completely nonresponsive to stimulation by TCR-derived signals. In one study (46), the $CD25^+$ cells remained unresponsive even when costimulatory signals were provided by addition of anti-CD28, while in another study (47), a modest response of the

$CD4^+CD25^+$ T cells was seen in the presence of anti-CD28. Most importantly, the $CD4^+CD25^+$ cells could adoptively suppress the responses of $CD4^+CD25^-$ cells in coculture studies (Figure 1). Suppression appeared to be mediated by a cell contact–dependent mechanism because suppression was not seen when the suppressors were separated from the responders by a semipermeable membrane, and supernatants from activated suppressors could not mediate suppression. Neutralizing antisuppressor cytokine antibodies (anti-IL-4, -10, TGF-β) alone, or in any combination, also failed to reverse the suppression. $CD4^+CD25^+$ T cells from IL-4-/- or IL-10-/- mice were as effective suppressors in vitro as $CD4^+CD25^+$ T cells from wild-type mice. This result should be compared with the effectiveness of these reagents in reversing the suppressive effects of $CD45RB^{low}$ cells in vivo (37, 39). Suppression in vitro required that the suppressor population be activated via the TCR. The antigen-specific proliferative response of TCR transgenic T cells was not suppressed by the $CD4^+CD25^+$ T cells, whereas the responses of the same cell mixture to anti-CD3 stimulation were completely suppressed.

No evidence was obtained that the suppression mediated by the $CD4^+CD25^+$ population was mediated by killing of the responder population by Fas/Fas-L–dependent mechanisms. The $CD4^+CD25^+$ T cells would respond to the combination of anti-CD3 + IL-2, and suppression in coculture studies could be overcome by the addition of IL-2 or by the enhancement of endogenous IL-2 production by anti-CD28. Nevertheless, the $CD4^+CD25^+$ population did not mediate suppression by binding and/or consuming IL-2 (functioning as an ''IL-2 sink''), as the suppressor cells completely inhibited IL-2 gene transcription and IL-2 production in the responder T cell population. No significant differences in the VαVβ T cell repertoire between the $CD25^+$ and $CD25^-$ populations could be defined, indicating that the $CD25^+$ population is not mono- or oligoclonal (47). The $CD25^+$ T cells did not express NK1.1, and they appear to be distinct from

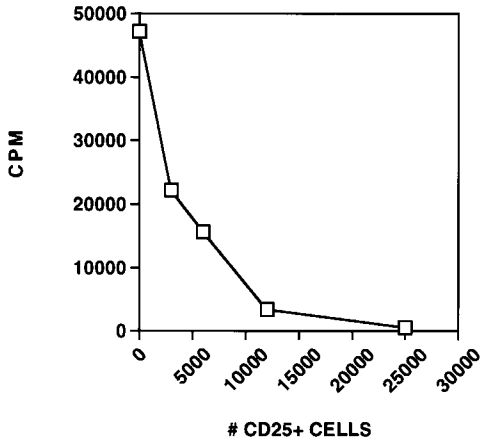


**Figure 1** $CD4^+CD25^+$ T cells are powerful suppressors of polyclonal T cell activation in vitro. $CD4^+CD25^-$ T cells (5 x $10^4$) were stimulated with soluble anti-CD3, T-depleted spleen cells, and different numbers of $CD4^+CD25^+$ suppressor cells. Almost complete suppression was observed when 12.5 x $10^4$ $CD4^+CD25^+$ cells were added (suppressor/responder = 1/4).

NK T cells. When compared with $CD4^+CD25^-$ T cells, the $CD25^+$ cells were similar in their expression of CD5, had a slightly higher proportion of $CD62L^{low}$ cells, and had a modest increase in the number of $CD69^+$ cells (5% –15%). They had an unusual pattern of expression of CD45RB; they lacked the $CD45RB^{high}$ population and were composed primarily $CD45RB^{int}$ and $CD45RB^{low}$ cells. Although these results suggested that the $CD25^+$ population might be heterogeneous and composed of a mixture of suppressor T cells and conventional memory T cells, we (47a) have been unable to identify a subpopulation of $CD25^+$ T cells that failed to manifest potent suppressor activity in vitro. We have, therefore, concluded that the $CD4^+CD25^+$ population represents a relatively homogeneous population of suppressor T cells.

While $CD25^+$ T cells from normal mice could not suppress antigen-specific responses, $CD4^+CD25^+$ T cells isolated from TCR transgenic mice on a conventional background could readily suppress the proliferative responses of TCR transgenic $CD4^+$CD25- T cells from the same mice. When TCR transgenic mice were bred to SCID mice and mice were selected that only expressed the transgenic TCR, the number of $CD4^+CD25^+$ T cells was decreased by >90% (13). Expression of the endogenous TCR α-chain was therefore required to generate the $CD4^+CD25^+$ population in TCR transgenic mice on a conventional background. One must therefore conclude that the true specificity of the $CD25^+$ population is defined by the endogeous receptor and not by the transgenic TCR. It is only fortuitous that ''antigen-specific'' suppression can be mediated by the transgene expressing $CD4^+CD25^+$ T cells. Once activated by antigen, suppressor effector function was completely antigen nonspecific. This was best illustrated when $CD4^+CD25^+$ T cells were propagated in culture by stimulation with anti-CD3 in the presence of IL-2. After 7 days of culture, the activated $CD25^+$ cells remain anergic to restimulation with anti-CD3 in the absence of IL-2; moreover, they had enhanced suppressor activity when cultured with fresh $CD4^+CD25^-$ T cells and anti-CD3. More importantly, the activated $CD25^+$ population suppressed antigen-specific responses of a wide variety of $CD25^-$ T cell populations derived from different TCR transgenic mice. There was no requirement for MHC restriction or antigen recognition for this suppression to occur (47a).

Even though these studies on the in vitro functions of the $CD4^+CD25^+$ cells reveal a potent suppressor activity, both the target cell and the mechanism by which suppression is mediated remain elusive. Some observations strongly suggested that the suppressor population acted on the APC. The $CD25^+$ cells readily suppressed the APC-dependent response to soluble anti-CD3, but not the response to the relatively APC-independent stimulus, plate-bound anti-CD3. Furthermore, some studies suggested that the suppressors may inhibit the generation or delivery of costimulatory signals, as suppression could be overcome either by the addition of exogenous IL-2 or by the generation of endogenous IL-2 by costimulation with anti-CD28. So far we have been unable to overcome suppression by addition of large numbers of fully activated APC, nor have we observed inhibition of expression of costimulatory molecules when APC were cultured in the presence of the

suppressor cells. Suppressor T cells functioned normally when fixed, activated APC were used in the cultures. Could the responder T cell be the target for suppression? This certainly remains an attractive possibility, but none of the studies by our group or others revealed a possible mechanism of such an activity. Although Fas/Fas ligand interactions do not play a role in suppression, it is possible that other known or unknown members of the TNF/TNFR family may be mediating the inhibition of cell growth in this system. Of course, it is still possible that we are dealing with a relatively labile, unknown, soluble suppressor factor.

What is the relationship between the $CD4^+CD25^+$ suppressor T cell population that functions in vivo and in vitro in a suppressor cytokine-independent manner and the $CD45RB^{low}$ T cells that mediate suppression of autoimmunity in vivo by secreting suppressor cytokines? It has recently been demonstrated (48) that the $CD45RB^{low}$ population can be subdivided based on expression of the CD38 antigen; approximately 50% of the $CD45RB^{low}$ cells express CD38. The $CD38^+$ population is about 50% $CD25^+$ and behaves in a similar fashion in vitro, i.e. it is anergic and suppressive. The $CD38^-$ population contained antigen-primed cells that were capable of mounting vigorous secondary responses in vitro and in vivo. Previous studies had shown that $CD45RB^{low}$ T cells were poor responders to stimulation via the TCR. Although these studies were interpreted as indicating that memory cells were more difficult to activate than naive cells, perhaps secondary to CD4-mediated downregulatory signals, it is much more likely that the poor responses of the $CD45RB^{low}$ cells to stimulation with anti-CD3 were secondary to active suppression mediated by the $CD38^+$ cells in that population. Both $CD25^+CD38^+$ and $CD25^+CD38^-$ T cells are anergic and suppressive (47a), so it remains possible that all of the suppressive activity of the $CD38^+$ population is mediated by the $CD25^+$ cells. Both the $CD38^+$ and $CD38^-$ subpopulations of the $RB^{low}$ pool were capable of inhibiting colitis in vivo. The suppressive capacity of $CD25^-CD38^+$ cells has not yet been evaluated in vivo or in vitro. It remains possible that multiple functional lineages of suppressor cells may be identified based on differential expression of these markers. It is puzzling that the in vitro suppressor activity of the $CD38^+$ cells was suppressor cytokine-independent, while their in vivo activity was cytokine-mediated.

## REGULATORY T CELLS IN OTHER AUTOIMMUNE DISEASES

Although this review has focused on autoimmune diseases that develop in animals from which regulatory T cells have been depleted, a considerable body of experimental data has been accumulated over the past 10 years which suggests that regulatory T cells are involved in almost all experimental animals models of autoimmunity. In the NOD mouse model of diabetes, a number of observations are compatible with a requirement for diminished regulatory T cell function for

transfer of disease. Diabetes could only be transferred from sick mice to normal syngeneic recipients if the recipients were fewer than 5 weeks of age (female) or 3 weeks of age (male). Furthermore, cyclophosphamide was required for induction of diabetes in young male as well as female NOD mice. Most importantly, $CD4^+$ T cells from nondiabetic NOD mice could prevent the transfer of diabetes from overtly diabetic mice into sublethally irradiated NOD recipients (49, 50). The protective cell population was not present in the spleen until 3 weeks of age and reached its highest activity at 8 weeks of age; suppressor cells were present in the thymus of neonates, which may explain why thymectomy at weaning accelerates disease. Diabetes could also be efficiently transferred to non-irradiated adult NOD recipients if they were Tx and $CD4^+$ T cell depleted. Depletion of $CD4^+$ T cells alone was not sufficient for disease transfer, and it was likely that Tx was needed to limit re-expansion of the $CD4^+$ regulatory T cells (51). Islet-infiltrating T cells from young nondiabetic mice could transfer diabetes to NOD/SCID mice, but cotransfer of $CD4^+CD45RB^{low}$ splenic T cells from the same mouse delayed the onset of disease (52). $CD4^+CD45RB^{low}$ T cells from overtly diabetic mice transferred disease and produced IFN-$\gamma$, while the protective $CD4^+CD45RB^{low}$ cells produced a Th2 or Th0 cytokine profile.

There is very little data as to the nature of the target antigen recognized by the regulatory T cells in NOD mice. Both $CD4^+$ (53, 54) and $CD8^+$ (55) T cell clones with suppressive activities have been isolated, but the antigens recognized by these clones have been very poorly characterized. In some cases the clones appeared to be autoreactive with self-MHC class II molecules (54), while in other cases both reactive to components in fetal calf serum and specifically reactive with islet cells (53). These clones produced a variety of cytokines including several uncharacterized suppressor factors, while other clones mediated their suppressive effects by producing TGF$\beta$ (54).

Although NK-like T cells have not been shown to play a regulatory role in the other models of autoimmunity described here, Gombert et al (56) demonstrated that NOD mice had a deficit in NK T cells, which was first seen at 3 weeks of age and persisted until 8 weeks of age. Both NK T cells in the thymus and the spleen lacked the ability to produce IL-4. It appears that NK T cells emerge from the thymus later in life in NOD mice, and this may explain why thymectomy at 3 weeks of age aggravates disease. NK T cells contained within the population of double negative thymocytes were capable of preventing the spontaneous onset of diabetes when transferred into prediabetic recipients (57). The protective effect of the transferred NK T cells was mediated by IL-4 and IL-10 because neutralization of IL-4 and IL-10 during the first week after NK T cell transfer inhibited protection by these cells. These studies have been interpreted as demonstrating that a deficiency of NK T cells in NOD mice contributes to the pathogenesis of IDDM by permitting the development of pathogenic Th1 effector cells. Preliminary studies suggest that a similar defect may be seen in human diabetes, as diabetic siblings had lower frequencies of $CD4^-CD8^-$ NK T cells that expressed the V$\alpha$24J$\alpha$Q TCR (58). NK T cell clones from the diabetic twins secreted only

IFN-γ upon stimulation, while clones from the at risk nonprogressor siblings and normals secreted both IL-4 and IFN-γ. As in the mouse, the loss of the capacity of NK T cells to produce IL-4 appears to be the major factor regulating disease susceptibility. Clearly, more studies are needed in both animal models and human to validate this hypothesis. Furthermore, it is not clear how the target antigens recognized by the NK T cells play a role in the pathogenesis of diabetes.

While one might have predicted that mice which expressed a transgenic TCR specific for an autoantigen would rapidly develop autoimmune disease, a number of studies have demonstrated that only a small percentage of mice which express a TCR specific for an autoantigen develop disease. Transgenic mice that expressed the α-and β-chains of an MBP-specific TCR exhibited only a low incidence of spontaneous EAE, which developed after 1 year of age. When T cells from these mice were stimulated in vitro they were not anergic; immunization of the transgenics with MBP peptide also resulted in the rapid development of EAE. When crossed to RAG-1 –/– mice, all the mice rapidly developed EAE spontaneously. It is likely that the resistance of mice that expressed the transgenic TCR on a conventional background was mediated by the low proportion of T cells expressing TCR encoded by the endogenous α- and β- TCR genes, which are not present in the RAG –/– mice (59, 60). When $CD4^+$ T cells from nontransgenic mice were transferred into the 3-week-old TCR transgenic RAG –/– mice, EAE onset was delayed, severity diminished, and the animals recovered from the disease. Recipients older than 45 days were less susceptible to protection. Crosses of TCR transgenic RAG-1 –/– mice with mice deficient in B cells, $CD8^+$ T cell, NK T cells, γ/δ T cells, or α/β T cells indicated that $\alpha/\beta^+$, $CD4^+$ T cells were the only cell population capable of mediating protection. Susceptibility of the mice to EAE correlated inversely with the diversity of their T cell repertoire. The nature of the target antigen recognized by the regulatory T cells in this model and the relationship of the regulatory T cells to the other types of regulatory cells described in this review remain to be determined. A similar protective effect of T cells that expressed receptors encoded by endogenous α- and β-chain genes was observed in mice that expressed a transgenic TCR specific for an pancreatic islet cell antigen (61).

## REGULATORY T CELLS ARE GENERATED IN THE THYMUS

In both the 3dTx model of induction of autoimmunity and the Tx-irradiation models, regulatory $CD4^+$ thymocytes can protect in reconstitution studies. In fact, $CD4^+$ thymocytes were considerably more protective in the rat Tx-irradiation diabetes model than were peripheral $CD4^+$ T cells (62). While fewer than $5 \times 10^6$ peripheral $CD4^+$ cells were not protective, significant protection was seen with $0.6 \times 10^6$ $CD4^+$ thymocytes. This simple finding raises a number of important questions about the potential antigens (s) that might be recognized by these

cells. It does not appear that regulatory T cells are generated by the antigen-driven expansion of a limited number of thymocyte precursors in the periphery. It remains possible that tissue-specific antigens may be expressed in thymus and that the regulatory T cells may be generated by recognition of such antigens on thymic epithelium, but the studies in the athyroid rat model (45) demonstrate that maintenance/expansion of the regulatory T cells also required the presence of the relevant autoantigen in the periphery as well as in the thymus.

Papiernik et al (63) were the first to demonstrate that $CD4^+CD25^+$ T cells originate in the thymus and are induced to express CD25 at the $CD4^+$ single positive stage. The $CD4^+CD25^+$ single positive thymocytes were not derived from $CD25^+$ double positive cells. Approximately 5% of $CD4^+$ thymocytes express CD25, while less than 0.3% of $CD8^+$ thymocytes express CD25 (64). The phenotype of the $CD4^+CD25^+$ thymocyte resembles that of the peripheral $CD4^+CD25^+$ T cells in that there is an enhanced expression of membrane activation markers. The capacity of $CD25^+$ T cells to migrate from the thymus to the periphery was studied after intrathymic injection of FITC. The percentage of $CD25^+$ cells within migrants and resident T cells was identical, suggesting that $CD25^+$ cells in the periphery can originate in the thymus. $CD4^+CD25^+$ thymocytes have the capacity for peripheral expansion, as shown by the transfer of $CD4^+$ cells to *nu/nu* recipients. Most importantly, $CD4^+CD25^+$ T cells were absent from the periphery and from the $CD4^+$ single positive thymocyte pool of IL-2–deficient mice. This finding indicates that IL-2 itself is important for the generation of these regulatory cells in the thymus and possibly for their maintenance/expansion in the periphery. It is still unclear how the expression of CD25 relates to this requirement for IL-2 stimulation. The thymocyte population also functionally resembled the $CD4^+CD25^+$ population found in the periphery since the $CD4^+CD25^+$ thymocytes were both anergic and suppressive. *Nu/nu* mice reconstituted with CD25-depleted adult thymocytes developed a wider spectrum of disease than that seen after d3Tx, including the development of signs of systemic autoimmunity.

Although studies of thymocyte differentiation over the past 10–15 years have offered major insights to the processes of positive and negative selection, it is still difficult to develop a model for the generation of regulatory T cells, particularly since their antigenic specificity is so ill defined. A number of animal models are now available that permit one to take a reductionist approach to the creation of models that might explain regulatory T cell differentiation in the thymus. The simplest view, based on the selective expression of the CD25 antigen at the $CD4^+$ single positive stage, is that regulatory T cells are generated during the process of negative selection. If the process of negative selection was inhibited, regulatory T cells would not be induced and autoimmunity might result. When the Keratin 14 promoter was used to re-express a class II MHC antigen in class II negative mice, the transgenic molecule was expressed only on thymic cortical epithelium; thymic medullary epithelium and bone marrow–derived cells were MHC class II

negative (65). Such mice lacked MHC class II expression on the critical cell types, which have been postulated to mediate negative selection. When $CD4^+$ T cells from these mice were cultured in vitro, they exhibited an enhanced autoreactivity, as measured by an elevated proliferative response in the syngeneic MLR and the generation of $CD4^+$ MHC class II–restricted cytolytic cells. These mice showed no evidence of autoimmunity since they did not express MHC class II antigens on their peripheral APC; however, when $CD4^+$ T cells from the mice were transferred into lethally irradiated syngeneic C57BL/6 mice, they induced acute graft-versus-host disease with bone marrow failure (66). Furthermore, these autoreactive $CD4^+$ T cells caused hypergammaglobulinemia and the production of autoantibodies when transferred into unirradiated C57BL/6 hosts. It cannot be determined from these studies if the autoreactivity resulted from the failure of deletion of high-affinity autoreactive T cells, from the failure to generate immunoregulatory cells, or a combination of both. It would be of interest to examine these mice as well as H2-M–deficient mice, which also exhibit a defect in the negative selection process (67), for the presence of $CD25^+$ anergic/suppressive T cells.

Could regulatory T cells be generated during the process of positive selection on thymic epithelium? Modigliani et al (68) have characterized a complex model in which an embryonic thymic rudiment from a day 10 fetus was transplanted onto a *nu/nu* recipient. This type of thymic epithelial (TE) graft is devoid of hematopoietic cells and was colonized by the host's hematopoietic cells with restoration of the T cell compartment. When TE was grafted to an allogeneic recipient, tolerance was induced to a variety of peripheral tissues of donor type that expressed nonthymic antigens (skin and heart). Transfer of high numbers of T cells from tolerant animals to athymic *nu/nu* recipients resulted in maintenance of the tolerant state in the adoptive host; the regulatory T cell that transferred tolerance was shown to be a $CD4^+$ T cell. The mechanisms whereby such regulatory T cells would suppress effector cells that recognized tissue-specific antigens have not yet been determined. This model system supports the view that the generation of regulatory T cells might occur at stages of the T cell differentiation process other than negative selection.

It is also possible that autoimmunity may result from defects in both positive and negative selection. Ridgway and Fathman (69, 70) have demonstrated that immunization of NOD mice with self-peptides resulted in an immune response to the self-peptide, with resultant autoproliferation of peripheral lymphocytes. NOD mice retained the capacity to respond normally to foreign peptides while demonstrating an abnormal response to self-peptides. The induction of autoreactivity was only seen in NOD mice, and homozygous expression of $I\text{-}Ag^7$ was required. It was proposed that the poor peptide binding properties of $I\text{-}Ag^7$ molecules were responsible for defective positive selection. Inefficient positive selection would require an increased affinity of the T cells to attain the requisite avidity needed for selection. These high-affinity T cells would then enter the periphery, and in collaboration with other genes they would mediate autoimmunity once an

inflammatory event broke self-tolerance. This model does not address the altered generation of regulatory T cells, but it is likely that the process of differentiation of regulatory T cells would also be abnormal and suboptimal.

## CONCLUDING REMARKS

Taken together, the potential contribution of disordered thymic selection to the generation of regulatory T cell function is a confusing, but important, area for future study. It is clear that manipulations of thymic architecture will likely result in the generation of both altered autoimmune effector cell function and in defects in regulatory T cell function. The evidence presented here strongly suggests that the regulatory $CD4^+$ cell lineage is unique and is a normal product of T cell differentiation in the thymus. I would like to propose a model to explain the generation of regulatory T cells under normal physiologic conditions. This model is heavily biased by the concept that the process of positive selection in the thymus is much less stringent that the process of negative selection; furthermore, self-antigens, which are expressed in or transported from the periphery to the thymus, play a very minor role in positive selection, but a critical role in negative selection. The experimental evidence to support this view is derived from experiments with MBP-deficient mice (71, 72). Such mice can generate a vigorous high-avidity T cell response to MBP, while the wild-type control strain is poorly responsive to this protein. First, it is clear that endogenous MBP is not required for positive selection. Second, the major function of self-antigens in the thymus is to induce clonal deletion of high-avidity T cells and to permit only the export from the thymus of low-avidity MBP-reactive T cells.

How then are regulatory T cells generated in the thymus? As is demonstrable in most immunologic systems, ''all or none'' phenomena are rare. I would propose that the process of negative selection may have outcomes other than simple clonal deletion or passage through to the periphery. Studies (73) with mature peripheral T cells and T cell clones have demonstrated that engagement of the TCR by so-called ''altered-peptide ligands'' may result in a permanent change in the effector functions of the clones. If the fit between the self-peptide/MHC complex and the TCR during negative selection is not of sufficient avidity to result in clonal deletion, yet not weak enough to allow the autoreactive T cells to pass through to the periphery, the outcome may fall into what we would term''altered negative selection'' (Figure 2). This process would result in a permanent change in the capacity of the self-reactive T cells to signal through the TCR. Cells surviving this process would leave the thymus in an incapacitated state: 1. They would not be able to differentiate into Th1 cells capable of producing organ-specific autoimmunity, 2. they might resemble the $CD4^+CD25^+$ cells and be completely anergic, 3. they might alternatively resemble the $CD45RB^{low}$ populations and produce suppressor cytokines, or 4. they may be unable to express membrane molecules which are critical for their differentiation into pathogenic Th1 effectors, such as the CD40

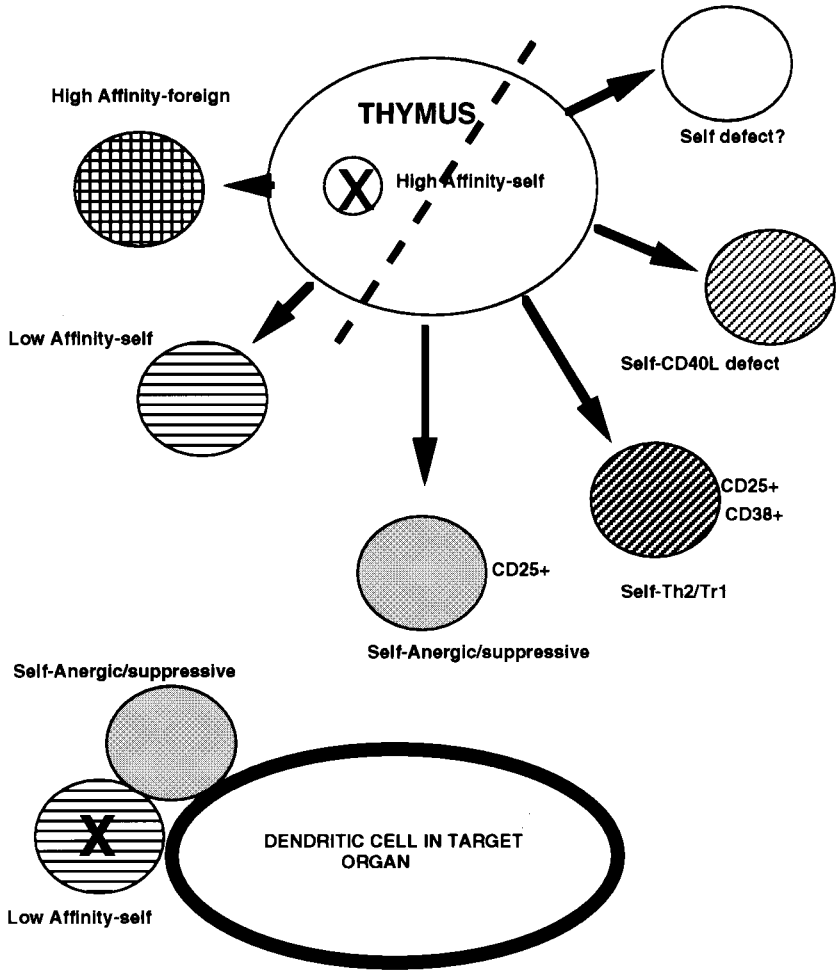


**Figure 2** The ''altered negative selection model'' for the generation of suppressor T cells. Suppressor T cells with a variety of functional phenotypes would be generated during the process of negative selection in the thymus because the fit between their TCR and the self-peptide/MHC complex is not optimal and results in a permanent change in the signaling capacity of their TCR. The precise mechanism by which they inhibit the activation of low-affinity anti-self effector cells on the surface of dendritic cells in the target organ remains to be defined.

ligand (38). A number of different functional phenotypes might be created that would account for the diversity of suppressor effector mechanisms described in this review.

What is the antigenic specificity of the regulatory T cells? As none of the studies we have described are really compatible with a model where the effectors and suppressors compete for the same peptide determinant of an antigen, I would favor the possibility that the suppressor populations are either specific for other peptide determinants on the target autoantigen or are specific for determinants on other peptides derived from proteins of the target organ. Once the pathologic process has been initiated by autoreactive effector cells, the organ-specific suppressors would home to the target organ, be activated by their target peptide, and mediate suppression. They might even be activated by the same APC that activates the effector cell, so a process of ''linked-suppression'' (74) would result. Although it is easiest to invoke a suppressor effector function that is mediated by a cocktail of suppressor cytokines (IL-4, -10, -13, and TGF-β), all the data on the $CD4^+CD25^+$ population suggest that a novel cell contact–dependent mechanism of effector T cell inactivation also plays a role.

Do regulatory T cells exist in humans and do they play a role in the pathogenesis of autoimmunity? Thus far, almost no information is available on the existence of subpopulations of $CD4^+$ T cells in humans that have any of the characteristics of the $CD4^+CD25^+$ or $CD4^+CD45RB^{low}$ cells that have been described in the mouse. We therefore focus on the possible implications of the findings in rodents to the pathogenesis and treatment of disease in humans. The studies by Sakaguchi et al (75) have emphasized that one potential drawback of a unique lineage of regulatory T cells is that they may be susceptible to a variety of environmental insults or genetic abnormalities. For example, while immunosuppressive therapy may be used to reduce or eliminate activated T cells, such treatment may actually induce autoimmune disease by also depleting regulatory T cells. Depletion of suppressor cells by different agents (drugs, radiation, infection, etc) might lead to development of autoimmune disease in the same organ system in a susceptible individual. The particular disease that would develop would be determined by the genetic background of the host.

Although the focus of this review has been on the role of regulatory T cells in controlling autoimmunity, it should be emphasized that regulatory T cells must have a much broader role in controlling immune responses. Do they also inhibit immune responses to foreign antigens? Our bias is that the majority of foreign antigen-specific T cells have much higher affinities than those autoreactive T cells which recognize organ-specific antigens; hence foreign antigen-reactive populations will be much less susceptible to inhibition. Needless to say, some members of the pool of foreign antigen–reactive T cells may have lower affinity receptors, and those cells may be susceptible to down-regulation by regulatory T cells. The most important class of antigens the response to which will be regulated by suppressor T cells will be the class of autoantigens involved in tumor immunity. Temporary depletion of regulatory T cell function might facilitate vaccination protocols to both tumor-specific antigens and weakly immunogenic pathogens. Lastly, an extension of this concept would involve attempts to enhance regulatory T cell function in patients with autoimmunity or in recipients of allografts.

Although most of the animal studies suggest that the regulatory T cells will be less effective as inhibitors of primed or activated T cells, they may still be capable of inhibiting relapses of chronic diseases by blocking epitope spreading and sensitization of new effectors.

In a timely, but scathing, editorial in 1988, G. Moller (76) summarized his reasons for questioning the existence of suppressor T cells. In 2000, there is little doubt that suppressor T cells play a critical role in regulating autoimmune disease in a large number of animal models. Although markers are now available that allow enrichment of suppressor T cell populations, they are still imperfect because all are also expressed on other cell types. More importantly, most of the properties of suppressor T cells that were poorly characterized in 1988—antigen-specificity, MHC restriction, frequency of reactive cells, mechanism of action, and cellular targets of suppression—remain so today. All of these topics should be the subject of fruitful investigations in the future.



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