Commentary

Certified Professionals: $CD4^+CD25^+$ Suppressor T Cells

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Although negative selection in the thymus and induction of anergy in the periphery have been widely accepted as mechanisms for controlling autoreactivity, much less attention has been devoted to the role of suppressor T cells in mediating dominant immunologic self-tolerance. In 1995, Sakaguchi et al. (1, 2) made the seminal observation that the transfer of $CD4^+$ T cells which had been depleted of the minor subpopulation (10%) of cells that coexpressed the IL-2 receptor (IL-2R) α-chain (CD25) to nu/nu recipients induced organ-specific autoimmune disease in the majority of recipients. Cotransfer of $CD4^+CD25^+$ cells with the $CD4^+CD25^-$ cells prevented the development of disease. The $CD4^+CD25^+$ population was also shown to be solely responsible for the prevention of autoimmunity observed after mice are thymectomized on the third day of life (3).

Rapid progress in the analysis of the regulatory function of the $CD4^+CD25^+$ T cell population was made by the development of in vitro model systems that mimicked the function of these cells in vivo (4–6). Although minor differences were observed in the results obtained by the different groups, it is widely accepted that these cells are both hyporesponsive and suppressive. Further studies demonstrated that the $CD4^+CD25^+$ T cells act through an APC-independent mechanism (7, and unpublished observations). Induction of suppressor activity requires that the $CD4^+CD25^+$ cells be activated through their TCR, but once activated, they suppress T cell activation in an antigen-independent manner without a requirement for reactivation through their TCR. Therefore, we proposed (4) that $CD4^+CD25^+$ cells represent a unique lineage of $CD4^+$ T cells that function as "professional suppressor cells." The publication in this issue of four papers dealing with different aspects of the function of $CD4^+CD25^+$ T cells clearly indicates that they have now received their "professional certification."

$CD4^+CD25^+$ T Cells Are Generated in the Thymus. Saoudi et al. (8) were the first to describe that potent T regulatory (Tr) cells were present in the thymus of the adult rat. PVG rats develop autoimmune diabetes after adult thymectomy and split-dose irradiation. Transfer of $CD4^+CD8^-$ thymocytes or $CD4^+CD45RC^-$ peripheral T cells prevented the development of disease. Subsequently, $CD4^+CD25^+$ T cells were identified in the mouse thymus (9) and shown by Itoh et al. (10) to resemble peripheral $CD4^+CD25^+$ T cells in their in vivo and in vitro suppressive capacities. Stephens and Mason (11) recently demonstrated that CD25 is also a marker for the $CD4^+$ T cells in the rat thymus that prevent autoimmune diabetes.

As the expression of CD25 appears during the transition of the $CD4^+CD8^+$ T cell to the $CD4^+CD8^-$ T cell (9), the generation of $CD4^+CD25^+$ T cells is part of normal thymocyte differentiation. We have proposed that (12) the induction of expression of CD25 (and perhaps other T cell activation antigens) reflects activation of this cell during a process that we have termed "altered negative selection." $CD4^+$ T cells that develop into $CD25^+$ T cells are likely to express a TCR with an intermediate affinity for self. This affinity is too low for negative selection, yet too high to allow the T cells to pass through to the periphery. As a result of this process of activation in the thymus, $CD4^+CD25^+$ T cells are rendered nonresponsive and suppressive.

Jordan et al. (13) have recently validated this hypothesis by demonstrating that T cells in mice expressing a high affinity transgenic TCR for influenza hemagglutinin (HA) as well as an HA peptide (under the control of SV40 early region promoter-enhancer sequences) developed into functional $CD4^+CD25^+$ suppressor cells. Radioresistant elements of the thymus played a critical role in generating the $CD4^+CD25^+$ T cells. In contrast, mice expressing an anti-HA TCR of much lower affinity did not develop into $CD25^+$ T cells. The authors of this study propose that $CD25^+$ T cells must express a TCR with a high affinity for the selecting peptide in order to be subsequently highly reactive with self-peptides they would encounter in the periphery. Although this double transgenic model offers a major insight into the mechanism of generation of the $CD25^+$ lineage, these experiments should be interpreted with caution as the self-antigen under study was widely expressed not only on all elements in the thymus, but in the periphery as well. The normal selection of $CD25^+$ T cells may involve other cellular elements in the thymus, such as bone marrow–derived dendritic cells (DCs), and may yield $CD25^+$ T cells expressing a broader spectrum of affinities.

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Identification of $CD4^+CD25^+$ T Cells in Man. Three papers in this issue (14–16) and three papers (17–19) appearing elsewhere now describe the properties of human $CD4^+CD25^+$ T cells. Given the obsession of most cellular immunologists with the concept of separating functionally distinct populations of cells based on their differential expression of membrane antigens, it is surprising that 6 yr elapsed between the identification of these cells in the mouse and the confirmation of their existence in man. One wonders if the human/clinical immunologists remained wary of the possible existence of "suppressor cells" based on the failure of biochemical and molecular studies to confirm their existence in the mouse in the early 1980's. In any case, there is marked agreement about the properties of human $CD4^+CD25^+$ T cells and it is somewhat reassuring that they resemble their murine counterpart in almost all of their critical properties.

Although most of these papers report that 5–15% of human $CD4^+$ T cells coexpress CD25, the sharp distinction observed between murine $CD25^+$ and $CD25^-$ T cells with the available mAbs and flow cytometry is not as evident in the human studies. Human $CD25^+$ T cells also have the phenotype of activated/memory T cells in that they are predominantly $CD45RO^+$ and a variable number also express MHC class II antigens. In one study (18), they were also shown to be $CD45RO^+$ and $CD45RB^{low}$ which is consistent with T cells which have been restimulated by antigen multiple times. The expression of CD122 (IL-2R β-chain) was easily detectable on human $CD25^+$ T cells, while its expression on murine $CD25^+$ cells is controversial. It is also notable that the frequency of $CD4^+CD25^+$ Tr cells is equivalent in the thymus of humans, rats, and mice (17). Thus, there appears to be remarkable evolutionary conservation across species in terms of the phenotype of the cell population responsible for T cell suppression in vivo. On the other hand, it is somewhat disappointing that none of these papers attempts a more detailed evaluation of the expression of some of the 260 CD markers now defined on human leukocytes, since CD25 is an imperfect marker that is expressed on every activated T cell. In view of the availability of multiple Abs to human chemokine receptors, an evaluation of their differential expression on human $CD25^+$ and $CD25^-$ T cells might have offered insights as to how these cells are mobilized to sites of inflammation.

All of the functional studies carried out with human $CD25^+$ T cells confirm what has been described with murine T cells. They are hyporesponsive to stimulation with anti-CD3, combinations of anti-CD3 and anti-CD28, and mature allogeneic DCs (mDCs). Alloantigen-activated $CD25^+$ T cells exhibited a cell cycle arrest in the G_1/G_0 phase. Human $CD25^+$ cells respond to TCR stimulation combined with IL-2, IL-4, and IL-15 although, even under these conditions, their proliferative responses do not approach those of similarly stimulated $CD25^-$ T cells. $CD25^+$ T cells were capable of suppressing the responses of $CD25^-$ cells to stimulation with anti-CD3 or alloantigen (14–16) presented by mDCs. Once activated, their suppressor function is nonspecific as was shown in studies of murine $CD25^+$ T cells (7). There is some variation among the reports as to the capacity of human $CD25^+$ T cells to produce either effector or suppressor cytokines. This may be secondary to the purity of the $CD25^+$ cells used in the assay and the specific assay (protein or mRNA). As has been reported with murine $CD25^+$ T cells (4, 6), human $CD25^+$ T cells appear to be capable of producing low levels of IL-10 and perhaps TGF-β, although equivalent levels of TGF-β mRNA were also detectable in stimulated $CD25^-$ T cells (16). Suppression in cocultures of $CD25^+/CD25^-$ T cells could be abrogated to some extent by the addition of IL-2, IL-4, or IL-15, alone or in combination. The extent of reconstitution of proliferation was not as complete as what has been observed in the mouse studies. All of the papers agree that suppression cannot be overcome by the addition of neutralizing Abs to IL-4, IL-10, IL-10R, or TGF-β.

As has been observed with murine $CD25^+$ T cells, all studies demonstrate that human $CD4^+CD25^+$ T cells express intracellular cytotoxic T lymphocyte–associated antigen (CTLA)-4 and expression is elevated after activation and culture. It is widely accepted that binding of CTLA-4 by its ligands results in a downregulation of T cell activation. However, it remains possible that this interaction might also lead to activation of certain T cell functions. The high level of expression of CTLA-4 on $CD25^+$ cells continues to remain an enigma. Different laboratories have reported disparate results. Takahashi et al. (20) claimed that the addition of anti–CTLA-4 or its F(ab′) fragment would reverse suppression in cocultures of murine $CD25^+/CD25^-$ cells. We have been unable to confirm these findings and have recently shown that induction of $CD25^+$-mediated suppressor function appears to be independent of costimulatory signals mediated by the interaction of either CD28 or CTLA-4 with their ligands, CD80/CD86 (unpublished observations). All of the human studies (14–19) fail to demonstrate reversal of suppression when anti–CTLA-4 or its F(ab′) fragment (19) is added. Nevertheless, the in vivo studies of both Takahashi et al. (20) and Read et al. (21) strongly implicate a role for CTLA-4 at some stage in the induction of $CD25^+$-mediated suppressor function during the pathogenesis of autoimmunity. One possibility is that, under some circumstances, engagement of CTLA-4 by its physiologic ligands or by Ab results in an inhibition of the TCR-derived signals needed for induction of suppressor function and the failure of the $CD25^+$ T cells to inhibit the development of autoimmune disease. This model is consistent with the accepted role of CTLA-4 in downregulating TCR-mediated activation of $CD4^+$ $CD25^-$ T cells.

Mechanism of Suppression. Taken together, all in vitro studies of murine and human $CD25^+$ T cells support a cell contact–dependent, cytokine-independent mechanism of suppression. Since suppression requires activation of the $CD25^+$ cells, it has been hypothesized that activation of these cells via their TCR induces a cell surface molecule(s) that mediates suppression by binding to a counter receptor

on the responder. The purported receptor on the $CD25^-$ T cell would also likely require induction by TCR-induced activation. Candidate receptors might include members of the TNFR superfamily or any cell surface antigen that expresses an intracellular immunoreceptor tyrosine–based inhibitory motif.

In contrast to the lack of involvement of cytokines in $CD25^+$-mediated suppression in vitro, IL-10, IL-4, and TGF-β have been implicated as mediating suppression of some (22–24), but not all (25, and unpublished observations), autoimmune diseases by Tr cells. While IL-10 is produced by the Tr cells (24), it has not yet been shown that IL-4 or TGF-β are actually produced by $CD25^+$ regulatory cells in vivo. Thus, it is difficult to conclude if these cytokines are responsible for the suppressive effects of the Tr cells or if they play a role in the differentiation of the Tr cells. Acceptance of a role for TGF-β or IL-4 as mediators of $CD25^+$-mediated suppression will require an analysis of the suppressive capacity of $CD25^+$ T cells from TGF-β/IL-4–deficient mice, as well as an analysis of the suppressability of $CD25^-$ T cells from mice which have genetic defects in their capacity to respond to these cytokines. It will also be important to determine if suppressive cytokines are only produced by a subpopulation of the Tr cells.

$CD4^+CD25^+$ T Cells and Transplantation Tolerance. Almost all in vivo studies of $CD4^+CD25^+$ T cells have focused on their role in preventing organ-specific autoimmune diseases. As the studies on the human $CD25^+$ T cells indicate that they are efficient suppressors of the mixed leukocyte reaction (MLR) in response to alloantigen presented by mDCs, it seems likely that they may also play a protective role in downregulating immune responses to alloantigen and preventing graft rejection. The paper by Taylor et al. (26) in this issue, a complementary paper from Hara et al. (27) in a recent issue of the Journal of Immunology, and an earlier paper by Jonuleit et al. (28) strongly support this view. Although these papers explore the role of Tr cells in three very different experimental models, I will attempt to point out the common threads in all three studies and offer a unified explanation for all the experimental results.

Taylor et al. (26, 29) have developed an in vitro model for the induction of transplantation tolerance by culturing C57BL/6 $CD4^+$ T cells with MHC class II incompatible bm12 stimulators in the presence of anti-CD40L or anti-CD80/CD86. After 10 d of culture, the recovered cells from the anti-CD40 ligand (CD40L)– or anti-CD80/CD86–treated cultures were poorly responsive upon in vitro stimulation and most importantly manifested a 30-fold reduction in their capacity to induce lethal GVHD with no additional immunosuppression in vivo. However, if $CD4^+CD25^+$ T cells were depleted from the responder population, the capacity of the responder cells to induce lethal GVHD was preserved. Thus, it appears that $CD25^+$ T cells in the responder population are critical for the induction of tolerance in vitro. Although it remains possible that the expression of the CD40L on the $CD25^+$ T cells may play a role in the induction of tolerance in vitro, an alternative explanation for these results is related to the differential requirements for costimulation for activation of the $CD25^+$ T cells compared with $CD25^-$ T cells. $CD25^+$ suppressor cells do not require costimulatory signals mediated by interactions of CD28/CTLA-4 with CD80/CD86 (unpublished observations) for induction of suppressor function, while activation of the effectors is likely to be strongly costimulation dependent. Thus, the culture conditions used in these experiments (anti-CD40L or anti-CD80/CD86) would favor suppression. The major consequence of $CD25^+$-mediated suppression is the induction of cell cycle arrest in the $CD25^-$ responders (7) and cell cycle arrest is normally followed by apoptotic cell death. As cell recovery was very low in the anti-CD40L–treated cultures, it is highly likely that tolerance in this model is secondary to deletion. This study also raises the issue of whether some of the therapeutic effects of anti-CD40L or anti-CD80/CD86 in other models are mediated by induction of $CD25^+$-mediated suppressor activity.

Wood's laboratory (27) has recently described an in vivo model that also defines a role for Tr cells, potentially derived from $CD4^+CD25^+$ T cells, in transplantation tolerance. Mice treated with a depleting anti-CD4 mAb in combination with donor alloantigen 28 d before transplantation accept donor, but not third party, grafts indefinitely. These mice are operationally tolerant to donor alloantigens as assessed by their ability to accept second heart or skin grafts from the same, but not third party donors, 100 d after transplantation. Tolerance can be adoptively transferred to naive syngeneic recipients resulting in the prolonged survival of heart grafts from the original donor, but not a third party. When $CD4^+$ T cells were isolated from mice rendered tolerant by this protocol and fractionated into RB^{high} and RB^{low} populations, the capacity to mediate transplantation tolerance was exclusively present in the $CD4^+CD45RB^{low}$ population. Similar results were observed with $CD25^+$ T cells from tolerant mice. Immune suppression transferred by the $CD45RB^{low}$ cells was mediated by IL-10 and not by IL-4. The Tr cells were able to suppress MLR's only when the alloantigen was presented via the indirect, but not the direct, pathway of allorecognition. Thus, the alloreactive regulatory $CD4^+CD45RB^{low}$ ($CD25^+$) T cells identified in this study resemble in many respects $CD4^+CD25^+$ Tr cells present in normal mice. It remains to be determined whether they are derived from resident $CD25^+$ T cells or from $CD25^-$ cells that are stimulated by alloantigen. In either case, I would propose that Tr cells are selectively activated because alloantigen is presented under conditions where costimulation may be limiting and antigen is chronically presented as donor-derived peptides in the context of host MHC class II molecules.

The studies of Jonuleit et al. (28) implicate the immature DCs (iDCs) as the candidate APCs involved in induction of suppressor T cell function. When cord blood lymphocytes were primed and repetitively stimulated with allogeneic iDCs, they expanded poorly. Furthermore, the recovered cells responded poorly to restimulation with mDCs, and suppressed the responses of naive cord blood cells to stimu-

lation by mDCs. Although the iDC-primed T cells produced IL-10, suppression was mediated by a cell contact–dependent mechanism. The Tr cells expressed high levels of CLTA-4, but Abs against CTLA-4 did not abrogate suppression. The Tr cells did not downregulate the capacity of mature DCs to stimulate and appeared to target the responder T cells. Thus, iDCs generated Tr cells have many phenotypic and functional properties in common with $CD4^+CD25^+$ resident Tr cells. As $CD4^+CD25^+$ T cells have been identified in cord blood, it will again be important to determine whether the regulatory cells are derived from that population or are generated from $CD25^-$ T cells.

The Tr cells generated by stimulation with iDCs in many respects resemble Tr1 cells (30) generated by stimulation of $CD4^+$ T cells in the presence of IL-10. Although both populations secrete IL-10 and inhibit stimulation by alloantigen in the MLR, Tr1 cells mediate suppression in vitro by secreting IL-10 and TGF-β. One explanation for this difference is that Groux et al. (30) used monocytes which are sensitive to the inhibitory effects of IL-10 as the stimulator population, whereas Jonuleit et al. (28) used mDCs that are resistant to the inhibitory effects of IL-10. Alternatively, differences in the culture techniques or the starting population (cord blood versus peripheral blood) of T cells may have resulted in the development of suppressor cells with distinct mechanisms of suppression. In any case, both of these studies are also consistent with the view that Tr cells can be selectively activated under conditions where costimulation is limiting. This unique property of Tr cells may permit them to be activated and then suppress immune responses to antigens presented by nonprofessional or poorly activated professional APCs.

Concluding Comments. Collectively, these studies form a solid foundation for further exploration of the role of $CD4^+CD25^+$ suppressor T cells in the regulation of both normal and pathological immune responses. One important theoretical question that must be addressed is the nature of the physiologic ligand recognized by the TCR expressed on $CD25^+$ T cells. A more practical, but equally important question, is how to expand these cells in vitro or in vivo for potential therapeutic purposes? Depletion of $CD4^+CD25^+$ T cells may become an important adjunct to the induction of immunity to tumor antigens or weak vaccines.

It is highly likely that thymic-derived $CD4^+CD25^+$ T cells represent only one of potentially multiple types of regulatory/suppressor T cells. As discussed above, chronic stimulation of $CD25^-$ T cells in vivo in the absence of costimulation may result in the production of Tr cells. Stephens and Mason (11) have described a population of $CD4^+CD25^-$ T cells that can prevent autoimmunity in the thymectomy/irradiation model in the rat, but only if recent $CD25^-$ thymic emigrants have been deleted from the population. Similarly, $CD4^+CD25^-$ T cells appear to be responsible for the resistance of mice expressing a transgenic TCR specific for myelin basic protein to the spontaneous development of autoimmune encephalomyelitis (31). As the expression of CD25 may not be stable, these $CD25^-$ suppressors may have been derived from $CD25^+$ T cells. Lastly, an intriguing possibility is that suppressors may be generated in vivo from fully differentiated Th1 effector cells by stimulation with antigen in the absence of costimulation in a manner similar to that seen in vitro with murine and human T cell clones (32).

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