

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 Further studies demonstrated that the CD4⁺CD25⁺ T cells act through an APC-independent mechanism (7, and unpublished observations).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. Saudi 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 G₁/G₀ 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 sup-

pressor 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-CD40L (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 alterna-

tive 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 CTLA-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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