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CUTTING EDGE

Cutting Edge: IL-2 Is Critically Required for the In Vitro Activation of $CD4^+CD25^+$ T Cell Suppressor Function

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$CD4^+CD25^+$ T cells are potent immunoregulatory cells that suppress TCR-induced proliferation of CD4 and CD8 T cells in vitro by a cell contact-dependent mechanism. Addition of IL-2 or anti-CD28 abrogates $CD4^+CD25^+$-mediated suppression of proliferation and has been assumed to "break suppression." We examined IL-2 mRNA by quantitative PCR in cocultures of mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Although IL-2 gene transcription was inhibited in the presence or absence of exogenous IL-2, the addition of anti-CD28 stimulated endogenous IL-2 production. Surprisingly, transcription of IL-2 mRNA was also restored in the cocultures in the presence of anti-IL-2. These results are most compatible with a model in which $CD4^+CD25^+$ T cells do not suppress the initial activation of $CD4^+CD25^-$ T cells, but mediate their suppressive effects following production of IL-2 by the responder cells resulting in both the expansion of the $CD4^+CD25^+$ T cells and the induction of their suppressor function. *The Journal of Immunology,* **2004, 172: 6519–6523.**

As potent immunoregulatory cells, $CD4^+CD25^+$ T cells not only suppress T cell proliferation in vitro, but also have the capacity to suppress immune responses to auto and alloantigens, tumor Ags, and infectious agents in vivo (1). The IL-2/IL-2R pathway is clearly important in the development and expansion of $CD4^+CD25^+$ cells in vivo as IL-2, IL-2Rα, and IL-2Rβ deficient ($-/-$) mice all die early in life of severe lymphoproliferation and autoimmune disease (2–4) and IL-2 and IL-2R$\beta^{-/-}$ mice have few or no $CD4^+CD25^+$ cells. Furthermore, while STAT5-deficient mice have very few $CD4^+CD25^+$ cells, mice transgenic for the active form of STAT5 possess a greater frequency of these cells (5, 6), thus confirming the requirement for IL-2 signaling in $CD4^+CD25^+$ T cell homeostasis. Recent studies suggest that the IL-2/IL-2R pathway is important for $CD4^+CD25^+$ T cells at several stages. $CD4^+CD25^+$ cell numbers are restored and the induction of autoimmune disease is prevented when an IL-2Rβ transgene is expressed solely in the thymus of IL-2R$\beta^{-/-}$ mice, indicating that an intact IL-2/IL-2R pathway is required in the thymus for generation of $CD4^+CD25^+$ cells (7). IL-2 signaling in the periphery is also required for the expansion of $CD4^+CD25^+$ cells. Although the transfer of wild-type $CD4^+CD25^+$ cells to IL-2R$\beta^{-/-}$ mice resulted in a marked expansion of the transferred cells and prevented the induction of disease, the transfer of wild-type $CD4^+CD25^+$ cells into IL-$2^{-/-}$ mice did not lead to engraftment or expansion of $CD4^+CD25^+$ cells and did not prevent autoimmune disease or death, suggesting that IL-2 is needed for the expansion and/or homeostasis of $CD4^+CD25^+$ cells. Similarly, Furtado et al. (8) demonstrated that $CD4^+$ T cells from IL-$2^{-/-}$ mice protected mice from spontaneous experimental autoimmune encephalomyelitis, while $CD4^+$ T cells from $CD25^{-/-}$ mice did not; thus, IL-2 derived from the recipient drove expansion of IL-$2^{-/-}$ cells, but was unable to drive the expansion of $CD4^+CD25^{-/-}$ cells. Other factors may also control the homeostasis of $CD4^+CD25^+$ T cells, as mice with defects in delivery or receipt of costimulation ($CD28^{-/-}$, CD80/$CD86^{-/-}$, $CD40^{-/-}$), may have quantitative or qualitative defects in $CD4^+CD25^+$-mediated functions (9, 10). Some of these defects appear to be independent of IL-2 (11).

Although IL-2 and its receptor clearly play a critical role in the homeostasis of $CD4^+CD25^+$ T cells in vivo, the potential contribution of IL-2 to their suppressive function remains elusive. Indeed, the molecular basis for suppression of T cell activation by $CD4^+CD25^+$ T cells in vitro is inhibition of IL-2 gene transcription in the $CD4^+CD25^-$ responder T cells. Furthermore, addition of IL-2 or the enhancement of costimulation by the addition of anti-CD28 are thought to break the anergic state of the $CD4^+CD25^+$ T cells and abrogate their suppressive function. One of the major problems with interpretation of this data is that T cell proliferation has been used as the major readout of suppression and both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells proliferate under these conditions. In this study, we have used a quantitative assay of suppression of IL-2 transcription to re-evaluate the role of IL-2 in the suppressor function of $CD4^+CD25^+$ T cells. We demonstrate that transcription of IL-2 mRNA remains fully suppressed in the presence of high concentrations of exogenous IL-2 and in the presence of proliferation of both the $CD4^+CD25^+$ suppressors and the $CD4^+CD25^-$ responders. In contrast, the addition of anti-CD28 to the suppression assay results in abrogation of suppression as transcription of

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IL-2 mRNA is partially restored. Thus, abrogation of the anergic state of CD4$^+$CD25$^+$ cells does not break suppression, as previously thought. Most importantly, the addition of anti-IL-2 completely abrogates the suppressive effects of CD4$^+$CD25$^+$ T cells on IL-2 mRNA transcription and demonstrates that IL-2 is not only required for the generation and peripheral maintenance of CD4$^+$CD25$^+$ cells, but is also required for the acquisition of suppressor function. The implications of these findings for the in vivo effects of CD4$^+$CD25$^+$ T cells are discussed.

Materials and Methods

Mice

Female BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). BALB/c Thy1.1 congenic mice were obtained from R. A. Seder (National Institutes of Health, Bethesda, MD) and were bred and maintained in National Institute of Allergy and Infectious Diseases/National Institutes of Health animal facilities.

Media, reagents, and Abs

Cells were grown in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated FCS, penicillin (100 μg/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (all Biofluids), and 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO). Biotin-anti-CD25 (7D4), PE-streptavidin, anti-CD3 (2C11), anti-CD28, and anti-IL-2 (S4B6) were purchased from BD PharMingen (San Diego, CA). Anti-CD90 (Thy1.2), anti-CD8, and anti-PE magnetic beads were purchased from Miltenyi Biotec (Auburn, CA). Human rIL-2 was obtained from the Preclinical Repository of the Biological Resources Branch, National Cancer Institute. IL-4 was purchased from R&D Systems (Minneapolis, MN). Flow cytometry analysis to assess cell purity was performed using CellQuest software (BD PharMingen).

Cell purification

CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells were purified as previously reported (12), unless stated otherwise. Purity ranged from 95 to 98%. T-depleted spleen cells (TΔS) were used as APC and were prepared by lysing erythrocytes with ACK lysis buffer (Biofluids), followed by depletion of CD90$^+$ cells on an autoMACS (Miltenyi Biotec). APC were irradiated with 3000R.

Proliferation assays

CD4$^+$CD25$^-$ cells (5×10^4), CD4$^+$CD25$^+$ cells (5×10^4), or CD4$^+$CD25$^-$ cells (5×10^4) cocultured with CD4$^+$CD25$^+$ cells (2.5×10^4) were cultured in 96-well plates (0.2 ml) with APC (5×10^4) and 0.25 μg/ml anti-CD3 for 72 h at 37°C/7% CO_2 in the presence of any indicated reagents. IL-2 was used at 50 U/ml, IL-4 at 20 ng/ml, and anti-CD28 at 0.5 μg/ml. Cultures were pulsed with [^{3}H]TdR for the last 6 h of culture. All experiments were set up in triplicate.

CFSE labeling

Thy 1.1 CD4$^+$CD25$^-$ or Thy 1.2 CD4$^+$CD25$^+$ cells were labeled with 2 μM CFSE for 8 min at room temperature. Cells were set up as in standard 96-well proliferation assays in triplicate. At 70 h, the triplicates were pooled and analyzed by flow cytometry.

Quantitative IL-2 mRNA analysis

CD4$^+$CD25$^-$ cells, CD4$^+$CD25$^+$ cells, or CD4$^+$CD25$^-$ cells cocultured with CD4$^+$CD25$^+$ cells were cultured with APC and 0.25 μg/ml anti-CD3 in the presence of indicated reagents for 44 h. Reactions were set up in 96-well plates as for the proliferation assays and the contents of one plate ($\sim 5 \times 10^6$ cells) pooled at 44 h. Total RNA was prepared with an RNeasy kit (Qiagen, Valencia, CA) during which on-column DNase I treatment (Qiagen) was performed. cDNA was made using Superscript II (Invitrogen, Carlsbad, CA) with random primers (Invitrogen). Primers and FAM-labeled probe for IL-2 and IL-4 were purchased from Applied Biosystems (Foster City, CA). As an internal control for normalization, 18S ribosomal RNA (rRNA) (Applied Biosystems) was used. Standard curves were set up for IL-2 and 18S rRNA. For each sample, the results of IL-2 gene expression were normalized relative to its 18s rRNA. The IL-2 gene expression of normalized unstimulated CD4$^+$CD25$^-$ cells was given an arbitrary value of 1.0 and the remaining samples were plotted relative to that value. All PCRs were performed in triplicate with a TaqMan Universal PCR Master Mix (Applied Biosystems). An ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used for 40 cycles of PCR. Each experiment was performed at least three times and a representative experiment is shown.

Results

CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells both proliferate when suppression is abrogated

As previously shown, coculture of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells results in inhibition of the proliferative response of CD4$^+$CD25$^-$ cells, which is reversed to varying extents by the addition of IL-2, IL-4, or anti-CD28 (Fig. 1*A*) (13–15). As purified CD4$^+$CD25$^+$ T cells proliferate when stimulated with anti-CD3 and IL-2 (13, 14), it has been assumed that when the in vitro nonresponsive state of the CD4$^+$CD25$^+$ cells is broken, their suppressive function is abolished. However, it is possible that CD4$^+$CD25$^+$ cells retain their suppressive function, and proliferation of both the CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ populations is driven by the addition of the exogenous agents. To address this possibility, we used CFSE dilution experiments to independently examine the proliferation of CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells under coculture conditions. As shown in Fig. 1*B*, CD4$^+$CD25$^-$ cells manifested a significant proliferative response which was enhanced by the addition of IL-2, IL-4, and anti-CD28. CD4$^+$CD25$^+$ cells cultured with anti-CD3 alone or with anti-CD3/CD28 did not

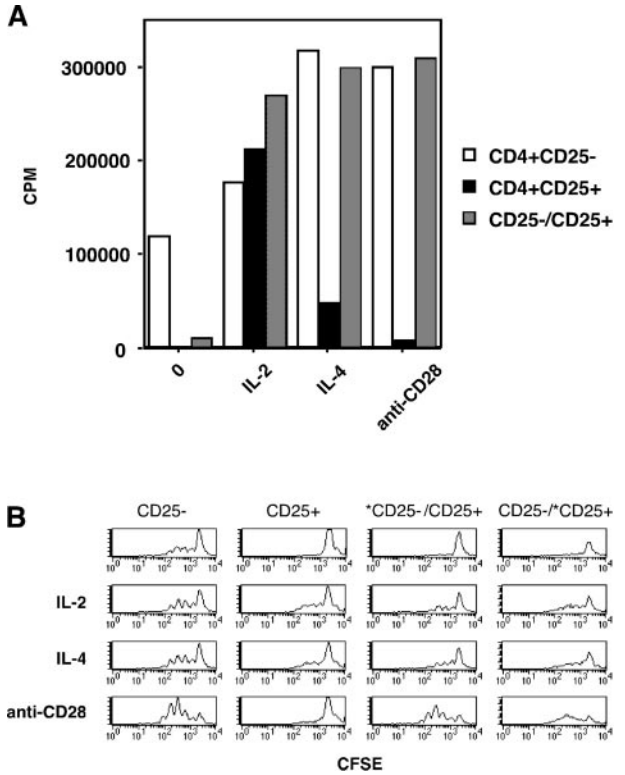

FIGURE 1. CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells proliferate and divide when suppression is abrogated. *A*, Cell-sorted CD4$^+$CD25$^-$ cells, CD4$^+$CD25$^+$ cells, or CD4$^+$CD25$^-$ cells cocultured with CD4$^+$CD25$^+$ cells, were stimulated with APC and anti-CD3 in the presence of IL-2, IL-4, or anti-CD28. Cultures were incubated for 72 h and pulsed with [^{3}H]TdR for the last 6 h of culture. *B*, Thy1.1 CD4$^+$CD25$^-$ cells, Thy 1.2 CD4$^+$CD25$^+$ cells, or Thy1.1 CD4$^+$CD25$^-$ cells cocultured with Thy1.2 CD4$^+$CD25$^+$ cells were stimulated with APC and anti-CD3 in the presence of IL-2, IL-4, or anti-CD28 for 64 h. Histograms were gated on Thy1.1-positive cells for CD4$^+$CD25$^-$ cells and Thy1.1-negative cells for CD4$^+$CD25$^+$ cells. CFSE-labeled cells in the coculture are marked with an asterisk.

divide significantly, but as was observed in the [^{3}H]TdR uptake assays, proliferated vigorously when stimulated with anti-CD3 and IL-2 or IL-4. In the cocultures, $CD4^+CD25^+$ cells markedly inhibited division of $CD4^+CD25^-$ cells. However, both the $CD4^+CD25^-$ and $CD4^+CD25^+$ populations divided when IL-2, IL-4, or anti-CD28 were added to the cocultures. Although the division of $CD4^+CD25^-$ cells led to distinct peaks of CFSE dilution, the division of $CD4^+CD25^+$ cells consistently showed a more diffuse pattern of CFSE dilution.

$CD4^+CD25^+$ cells inhibit IL-2 mRNA in the presence of IL-2 and IL-4, but not anti-CD28

Although both $CD4^+CD25^-$ and $CD4^+CD25^+$ cells proliferated in the presence of exogenous IL-2, IL-4, and anti-CD28, the possibility remained that the $CD4^+CD25^+$ cells remained functional with respect to their ability to inhibit IL-2 mRNA. When measured by quantitative PCR (qPCR),[3] maximum levels of IL-2 mRNA were observed under these limiting culture conditions after 44 h of stimulation (data not shown). Fig. 2 is divided into two parts with the results of the [^{3}H]TdR uptake assay on the left side and the corresponding qPCR analysis of the same cell populations on the right. In agreement with our previous Northern analysis (14), the transcription of IL-2 mRNA in $CD4^+CD25^-$ cells was inhibited in the presence of $CD4^+CD25^+$ cells (Fig. 2*A*). When IL-2 (Fig. 2*A*) or IL-4 (Fig. 2*B*) were added to the cultures, the levels of IL-2 mRNA were not significantly different in $CD4^+CD25^-$ cells stimulated in the absence of $CD4^+CD25^+$ cells and were never induced in $CD4^+CD25^+$ cells alone. However, IL-2 mRNA (Fig. 2*A*) and IL-4 mRNA (data not shown) were still inhibited. Thus, the proliferative response detected by [^{3}H]TdR uptake and CFSE dilution was solely driven by the exogenous cytokines as $CD4^+CD25^+$ cells were functional and suppressed IL-2 mRNA. In these experiments, $CD4^+CD25^-$ cells were not purified after coculture with $CD4^+CD25^+$ cells and it was possible that the decrease in IL-2 mRNA was a result of dilution from the additional cells. However, $CD4^+CD25^+$ cells comprise 33% of the sample while IL-2 mRNA inhibition was always greater than 80%. Furthermore, in preliminary experiments not depicted, greater than 75% inhibition of IL-2 mRNA was observed when $CD4^+CD25^+$ cells comprised only 20% of the coculture.

The addition of anti-CD28 also abrogated $CD4^+CD25^+$ T cell-mediated suppression (Fig. 2*C*, *left panel*), however, anti-CD28 induces transcription of IL-2 (16). The addition of anti-CD28 to $CD4^+CD25^-$ cells alone increased IL-2 mRNA by 2-fold, but had little effect on IL-2 mRNA in $CD4^+CD25^+$ cells alone (Fig. 2*C*, *right panel*). However, in cocultures stimulated in the presence of anti-CD28, IL-2 mRNA was restored to levels comparable to $CD4^+CD25^-$ cells alone. The experiment in Fig. 2*C* was repeated a number of times and in the presence of anti-CD28 the increase in IL-2 mRNA levels ranged between 50 and 100% of the levels observed in $CD4^+CD25^-$ cells alone. IL-4 mRNA levels were increased to ~60% of $CD4^+CD25^-$ cells alone as well (data not shown). Thus, the addition of anti-CD28 partially abrogates the suppressive effect of the $CD4^+CD25^+$ T cells on IL-2 mRNA transcription in the $CD4^+CD25^-$ responders.

[3] Abbreviations used in this paper: qPCR, quantitative PCR; GITR, glucocorticoid-induced TNFR.

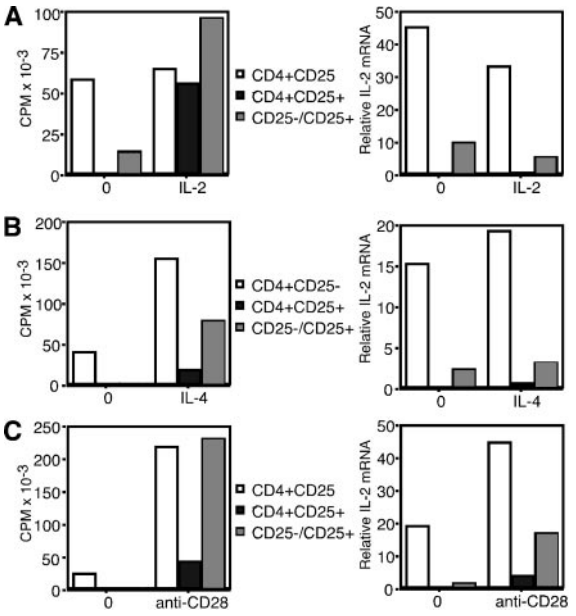

FIGURE 2. Quantitative IL-2 mRNA analysis. $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells, or $CD4^+CD25^-$ cells cocultured with $CD4^+CD25^+$ cells, were stimulated with APC and anti-CD3 in the presence of (*A*) IL-2, (*B*) IL-4, or (*C*) anti-CD28. Cultures were incubated for 72 h and pulsed with [^{3}H]TdR for the last 6 h of culture (*left panels*) or cells were harvested at 44 h, mRNA was isolated, and qPCR was performed (*right panels*). Samples were normalized to 18S rRNA and a relative value of 1.0 was given to unstimulated $CD4^+CD25^-$ cells.

$CD4^+CD25^+$ cells require IL-2 to inhibit IL-2 transcription in $CD4^+CD25^-$ cells

As recent in vivo experiments have strongly suggested that IL-2 is required for the generation and function of $CD4^+CD25^+$ T cells (17), we examined IL-2 mRNA in our coculture system in the presence of anti-IL-2 to determine whether IL-2 was also required for the activation of suppressor function. Although the addition of anti-IL-2 completely blocked the proliferation of $CD4^+CD25^-$ cells alone (Fig. 3*A*), the addition of anti-IL-2 did not block the transcription of IL-2 mRNA (Fig. 3*B*), allowing us to examine the effect of anti-IL-2 on $CD4^+CD25^+$ function. The addition of anti-IL-2 to the coculture restored IL-2 mRNA (Fig. 3*B*). Thus, a certain level of IL-2 production by $CD4^+CD25^-$ cells appears to be required to induce $CD4^+CD25^+$-mediated inhibition of IL-2 mRNA production. As IL-4 in the presence of a TCR signal can stimulate proliferation of $CD4^+CD25^+$ cells and induce $CD4^+CD25^+$ suppressor function (15), we also examined whether IL-4 could substitute for IL-2 in the induction of $CD4^+CD25^+$-mediated suppression. In the presence of IL-4 alone, the results were similar to those shown in Fig. 2*B* (data not shown). However, when IL-4 was added to the cocultures in the presence of anti-IL-2, IL-2 mRNA was not restored, indicating that IL-4 can substitute for IL-2 in vitro in inducing $CD4^+CD25^+$ suppressor function.

Discussion

Using a qPCR protocol for IL-2 mRNA, we have established that $CD4^+CD25^+$ cells suppress transcription of IL-2 mRNA in their target cells and that suppression of IL-2 mRNA is not abrogated under conditions in which the proliferation of

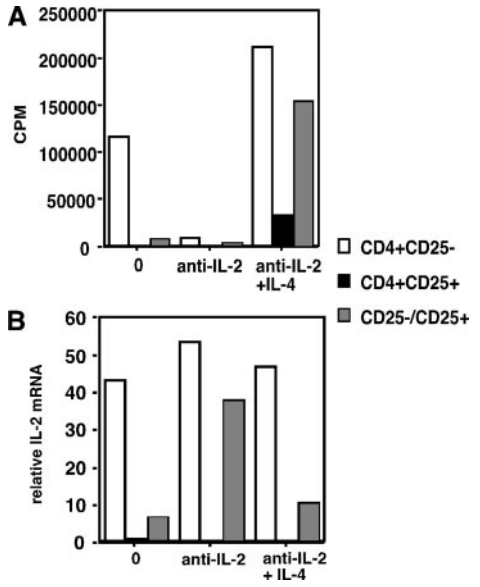

FIGURE 3. Activation of $CD4^+CD25^+$ T cell suppressor function requires IL-2. $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells, or $CD4^+CD25^-$ cells cocultured with $CD4^+CD25^+$ cells, were stimulated with APC and anti-CD3 in the absence or presence of anti-IL-2 (10 μg/ml) or anti-IL-2 plus IL-4. *A*, Cultures were incubated for 72 h and pulsed with [^{3}H]TdR for the last 6 h of culture. *B*, Cells were harvested at 44 h, mRNA was isolated, and qPCR was performed. Samples were normalized to 18S rRNA and a relative value of 1.0 was given to unstimulated $CD4^+CD25^-$ cells.

$CD4^+CD25^+$ T cells is driven by a TCR signal and high concentrations of exogenous IL-2 or IL-4. As these in vitro data demonstrate that suppressive function is not abrogated when anergy is broken, suppressor function may be maintained during the Ag-specific proliferation of $CD4^+CD25^+$ T cells in vivo. This result also rules out competitive absorption of IL-2 as a major factor in the suppressive function of $CD4^+CD25^+$ T cells. In contrast, the addition of anti-CD28 resulted in the abrogation of $CD4^+CD25^+$ T cell-mediated suppression of IL-2 mRNA. As the levels of IL-2 mRNA are never restored to that seen when $CD4^+CD25^-$ cells alone are stimulated with anti-CD3/CD28, it remains possible that some suppression of IL-2 production still occurs. It has been previously demonstrated that the addition of excess numbers of LPS-activated B lymphocytes, that expressed very high levels of CD80/CD86, had very little effect on reversing the suppressive effects of $CD4^+CD25^+$ T cells in vitro (12, 18). Therefore, the reversal of suppression seen with anti-CD28 in vitro may be secondary to the potent effects of anti-CD28-mediated costimulation on the induction of endogenous IL-2 production. In contrast, George et al. (18) have demonstrated that $CD4^+CD25^-$ cells escape $CD4^+CD25^+$-mediated suppression when stimulated with activated DCs. The physiologic significance of these observations is that they support the notion that there must be a balance between suppression and activation of immune responses. Thus, $CD4^+CD25^+$ cells exert their suppressive function under low costimulatory/low Ag conditions such as those that would be expected from autoantigens, but under the appropriate conditions, such as those provided by activated DC, effector cells escape suppression and are able to mount an appropriate and robust immune response to pathogens.

A clear role for IL-2 in the generation, maintenance, and expansion of $CD4^+CD25^+$ cells in vivo has been convincingly established (17). The only exception to this is the observation that $CD4^+CD25^+$ T cells from mice that express the IL-2Rβ chain only in the thymus, and cannot respond to IL-2 in the periphery, exist in normal numbers and are capable of suppressing the development of the autoimmune syndrome that normally develops in IL-2Rβ chain-deficient mice. However, it remains possible that the output of these cells from the thymus is abnormally high secondary to the transgenic expression of the IL-2Rβ chain and that they retain their capacity to suppress for a certain period of time after receiving the IL-2 signal in the thymus. Their failure to survive and suppress autoimmunity upon transfer to IL-$2^{-/-}$ mice is consistent with this scenario.

We have demonstrated here, for the first time, that IL-2 is also required for the activation of suppressor function of $CD4^+CD25^+$ T cells in vitro and that IL-4 can substitute for IL-2 in this process. An alternative explanation for this result is that IL-2/IL-4 function only as critical survival factors for $CD4^+CD25^+$ T cells in vitro. We believe this explanation is unlikely as we have recently demonstrated (15) that activation of suppressor function during preculture of $CD4^+CD25^+$ cells before their coculture with $CD4^+CD25^-$ cells also required the presence of IL-2, yet no difference in the recovery of cells at 1 or 2 days was observed in the presence or absence of IL-2. In the present study, IL-2 mRNA was measured at 40 h of culture, when the effects of IL-2 on cell recovery were minimal.

It might be regarded as paradoxical that the action of one or the other of the two major T cell growth factors is required for subsequent suppression of their own production. However, the initial activation of effector $CD4^+CD25^-$ T cells with resultant production of IL-2/IL-4 may be needed for regulation of the function of $CD4^+CD25^+$ T cells for two distinct purposes. First, it may facilitate the nonspecific expansion of the $CD4^+CD25^+$ T cells in situ at the site of the response. Although $CD4^+CD25^+$ T cells are nonresponsive to IL-2/IL-4 alone, they express high levels of the glucocorticoid-induced TNFR (GITR) and engagement of the GITR by an agonistic mAb (19) or its ligand[4] in the presence of IL-2 or IL-4 results in their proliferation in vitro. Recently, it has been shown that the GITR-L is expressed at high levels on resting APC, particularly B lymphocytes (20, 21). Second, the requirement for initial activation of effector cells before induction of suppressor function imposes a time constraint on when suppression can become manifest. Indeed, it would be highly desirable in the immune response to infectious agents that a certain level of effector function be established before induction of suppressor function which subsequently would lead to a down-modulation of effector function and the prevention of an overexuberant immune response (22).

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