Cutting Edge: Control of CD8$^+$ T Cell Activation by CD4$^+$CD25$^+$ Immunoregulatory Cells

Ciriaco A. Piccirillo and Ethan M. Shevach[1]

CD4$^+$CD25$^+$ regulatory T cells inhibit organ-specific autoimmune diseases induced by CD4$^+$CD25$^-$ T cells and are potent suppressors of CD4$^+$CD25$^-$ T cell activation in vitro. We demonstrate that CD4$^+$CD25$^+$ T cells also suppress both proliferation and IFN-γ production by CD8$^+$ T cells induced either by polyclonal or Ag-specific stimuli. CD4$^+$CD25$^+$ T cells inhibit the activation of CD8$^+$ responders by inhibiting both IL-2 production and up-regulation of IL-2Rα-chain (CD25) expression. Suppression is mediated via a T-T interaction as activated CD4$^+$CD25$^+$ T cells suppress the responses of TCR-transgenic CD8$^+$ T cells stimulated with soluble peptide-MHC class I tetramers in the complete absence of APC. These results broaden the immunoregulatory role played by CD4$^+$CD25$^+$ T cells in the prevention of autoimmune diseases, but also raise the possibility that they may hinder the induction of effector CD8$^+$ T cells to tumor or foreign Ags. ***The Journal of Immunology,*** **2001, 167: 1137–1140.**

Studies in a number of experimental models have demonstrated the existence of regulatory T cell populations that prevent the activation of autoreactive T cells (1–3). The most useful marker to date for identification of regulatory T cells is the CD25 (IL-2Rα-chain) Ag that is present on 5–10% of CD4$^+$ T cells in normal animals (4–8). The functional properties of murine CD4$^+$CD25$^+$ T cells have been extensively studied in vitro. They demonstrate profound anergy to stimulation via their TCR, and this anergic state cannot be reversed by costimulation with anti-CD28 (9, 10). More importantly, when CD4$^+$CD25$^+$ T cells are cocultured with CD4$^+$CD25$^-$ T cells, they induced profound suppression of T cell activation by down-regulating IL-2 production in the responding CD4$^+$CD25$^-$ T cells (10). The suppressive activity of the CD4$^+$CD25$^+$ T cells requires that they be activated via their TCR and is cell contact dependent but cytokine independent (10, 11).

The mechanism by which CD4$^+$CD25$^+$ T cells mediate their suppressive effects is poorly understood. The physiologic ligand recognized by their TCR is unknown, and considerable controversy exists as to their cellular target. Thornton and Shevach (12) have demonstrated that the suppressors do not modulate APC function, whereas other laboratories have raised the possibility that they act by suppressing APC function (13) or by competing for APC-derived costimulatory signals (9). The potential suppressive activity of CD4$^+$CD25$^+$ T cells on non-CD4$^+$ responder cells has not been studied in detail. Here, we demonstrate that CD4$^+$CD25$^+$ T cells suppress CD8$^+$ T cell proliferation and IFN-γ production induced by polyclonal or Ag-specific stimuli. In addition, the effects of the CD4$^+$CD25$^+$ T cells on CD8$^+$ cells are more complex than their effects on CD4$^+$CD25$^-$ responders, because they suppress both IL-2 production and CD25 expression. Finally, we made use of peptide-MHC tetramers to stimulate CD8$^+$ responders in a two-cell suppressor assay system to formally demonstrate that CD4$^+$CD25$^+$ cells mediate their suppressor function via a T-T cell interaction and in the absence of APC.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (specific for $OVA_{257–264}$ peptide), F5 (specific for nucleoprotein $(NP)_{366–374}$ influenza peptide), and P14 (specific for lymphocytic choriomeningitis virus (LCMV) gp33–41 peptide) CD8$^+$ TCR-transgenic (Tg) mice were obtained from Taconic Farms (Germantown, NY). All mice used were 6–12 wk of age.

The mAbs

The following Abs were used for flow cytometry experiments: biotin-anti-CD25 (7D4 clone), FITC-streptavidin, PE-anti-CD4, PE-anti-CD8, FITC-anti-CD25, FITC-anti-CD69, and purified anti-CD3ϵ (2C11), all of which were purchased from BD PharMingen (San Diego, CA).

Peptides

OT-I $OVA_{257–264}$, F5 $NP_{366–374}$, and LCMV gp33–41 peptides were provided by R. Germain and J. Yewdell (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Peptides were synthesized and purified by the Laboratory of Molecular Structure Peptide Synthesis Laboratory (National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Tetramers

MHC class I H-2K^b-$OVA_{257–264}$ tetramer solutions were prepared at the National Institute of Allergy and Infectious Diseases tetramer facility.

Cell purification and culture

CD4$^+$CD25$^+$ T cells were isolated on a FACStar cell sorter (BD Biosciences, San Jose, CA) as described previously (10). The purity of the final CD4$^+$CD25$^+$ preparation was typically >95%. T-depleted spleen cells (10; TdS) were irradiated at 3000 rad and pulsed for 30 min at 37°C with

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Received for publication April 30, 2001. Accepted for publication June 4, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Ethan M. Shevach, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N311, 10 Center Drive, Bethesda, Maryland 20892-1892. E-mail address: ems1@mail.nih.gov

[2] Abbreviations used in this paper: NP, nucleoprotein; LCMV, lymphocytic choriomeningitis virus; Tg, transgenic; TdS, T-depleted spleen cells.

0022-1767/01/$02.00

an appropriate peptide. Activated CD4$^+$CD25$^+$ cells were prepared as previously described (12). Briefly, cell-sorted CD4$^+$CD25$^+$ cells were cultured with irradiated APC (1:1 ratio), anti-CD3 (0.5 μg/ml), and human IL-2 (5 ng/ml, 100 U/ml) for 72 h and were then split and maintained in IL-2 medium for ~7–14 days. CD8$^+$ T cells were purified either by negative (depletion of B220-, CD4-, and I-A^b-positive cells) or positive selection (using CD8α magnetic beads) on the AutoMACS magnetic separation system (Miltenyi Biotec, Auburn, CA). For experiments involving tetramer stimulation, OT-I CD8$^+$ T cells were FACS purified using Abs against Thy1.2 and CD8α molecules, with final purities of >99%.

Proliferation assays

Proliferation assays were performed by culturing CD8$^+$ T cells (5×10^4) in flat-bottom microtiter plates (0.2 ml) with peptide-pulsed APC ($1–2 \times 10^5$) and resting or activated CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells for 72 h at 37°C in complete medium (10). Human rIL-2 was purchased from Peprotech (Rocky Hill, NJ). Cell cultures were pulsed with [^{3}H]TdR for the last 8 h. All data represent the average cpm of triplicate determinations. All proliferation experiments were repeated at least three times.

For cytokine production, supernatants were taken at 72 h, and the production of IFN-γ was measured using an ELISA kit (R&D Systems, Minneapolis, MN).

Flow cytometry

Cells were collected and stained with PE-CD8α and FITC-CD69 or FITC-CD25 and analyzed with a FACScan flow cytometer (BD Biosciences).

Results and Discussion

When freshly explanted CD4$^+$CD25$^+$ T cells from normal mice were cocultured with F5 TCR-Tg CD8$^+$ T cells, significant cell-dose-dependent suppression of proliferation was observed with soluble anti-CD3 as the stimulus in the presence of APC (Fig. 1*A*). When the TCR-Tg cells were stimulated with specific peptide, no inhibition of proliferation was observed (data not shown). When activated CD4$^+$CD25$^+$ T cells from normal mice were used, marked suppression of proliferation of F5 CD8$^+$ TCR-Tg T cells was observed with peptide stimulation (Fig. 1*C*). This finding confirms previous studies (12) using CD4$^+$ T cells as responders in which the CD4$^+$CD25$^+$ T cells required activation via their TCR to manifest suppressor function, but following activation, suppressor effector function was Ag-nonspecific and did not require restimulation of the suppressors via their TCR.

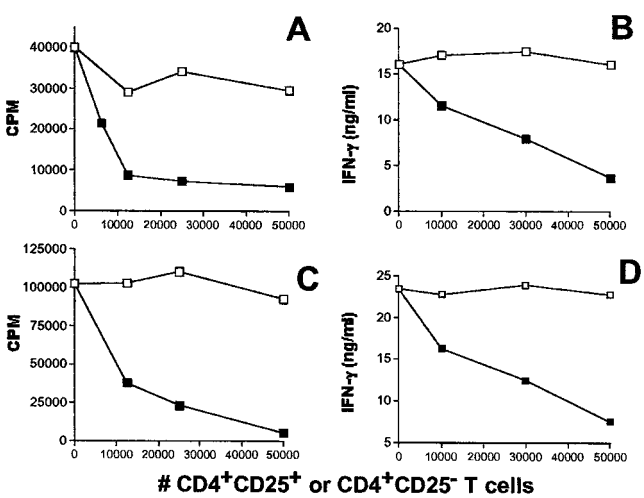

FIGURE 1. CD4$^+$CD25$^+$ T cells suppress CD8$^+$ T cell proliferation and effector function. Tg F5 CD8$^+$ T cells (5×10^4) were stimulated with anti-CD3 and TdS (2×10^5; *A* and *B*) or NP$_{366–374}$ peptide-pulsed TdS (2×10^5; *C* and *D*) and in the presence of either freshly isolated (*A* and *B*) or activated (*C* and *D*) CD4$^+$CD25$^+$ (■) or CD4$^+$CD25$^-$ (□) T cells. Proliferation (*A* and *C*) and IFN-γ production (*B* and *D*) were measured. Results from a representative experiment are shown.

Because CD8$^+$ T cells will produce significant amounts of IFN-γ in the absence of previous priming, we next studied the effects of CD4$^+$CD25$^+$ T cells on the capacity of CD8$^+$ T cells to produce IFN-γ. Freshly explanted CD4$^+$CD25$^+$ T cells readily suppressed IFN-γ production by CD8$^+$ T cells stimulated with anti-CD3 (Fig. 1*B*), and activated CD4$^+$CD25$^+$ T cells suppressed IFN-γ production when the CD8$^+$ T cells were stimulated with specific peptide (Fig. 1*D*). We have consistently shown >50% suppression of both proliferation and IFN-γ secretion at a CD25$^+$: CD8 ratio of 1:2, and >75% suppression at a 1:1 cell ratio. CD4$^+$CD25$^+$ T cells (Fig. 1, *A* and *C*) cultured alone do not proliferate and do not secrete IFN-γ (data not shown). CD4$^+$CD25$^-$ T cells (Fig. 1*C*) cultured in the absence of CD8$^+$ T cells do not produce IFN-γ (data not shown).

IL-2 will reverse suppression when CD4$^+$CD25$^+$ suppressors are cocultured with CD4$^+$CD25$^-$ responders. It has been proposed that IL-2 can directly act on the suppressors and reverse their anergic phenotype and consequently disable their suppressive capability (14). Alternatively, the addition of exogenous IL-2 may simply be circumventing the block in IL-2 production induced in the responders by the CD4$^+$CD25$^+$ suppressors. When fresh CD4$^+$CD25$^+$ T cells (Fig. 2*A*) or activated CD4$^+$CD25$^+$ T cells (Fig. 2*B*) were cocultured with OT-I CD8$^+$ T cells, significant suppression of proliferation (~60%) and IFN-γ production (Fig. 2*C*) was observed with soluble anti-CD3 or peptide stimulation.

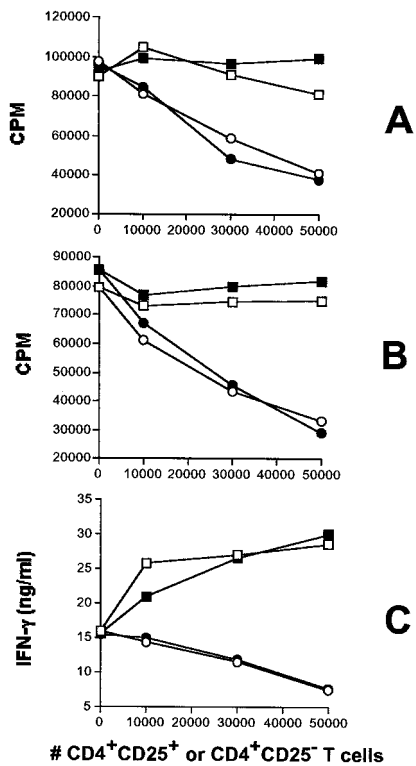

FIGURE 2. IL-2 does not reverse CD4$^+$CD25$^+$-mediated suppression. *A*, Tg OT-I CD8$^+$ T cells (5×10^4) were stimulated with anti-CD3 (0.5 μg/ml) and TdS (2×10^5) and either freshly isolated CD4$^+$CD25$^+$ (circles) or CD4$^+$CD25$^-$ (squares) T cells in the presence (filled symbols) or absence (open symbols) of exogenous IL-2 (100 U/ml). *B*, Tg OT-I CD8$^+$ T cells (5×10^4) were stimulated with OVA$_{257–264}$ peptide-pulsed TdS (2×10^5) and either activated CD4$^+$CD25$^+$ (circles) or CD4$^+$CD25$^-$ (squares) T cells in the presence (filled symbols) or absence (open symbols) of exogenous IL-2 (100 U/ml). Proliferation was assayed as in Fig. 1*C*. Supernatants from *B* were collected at 72 h, and IFN-γ ELISA was performed.

Suppression was not reversed by the addition of exogenous IL-2 at all suppressor:responder ratios or by enhancement of endogenous IL-2 production by the addition of anti-CD28 (data not shown). Increasing the amount of IL-2 added to the cocultures to 100 U/ml also had no effect. It should be noted that the proliferative response of $CD4^+CD25^+$ T cells to IL-2 is modest when compared with activated $CD4^+CD25^-$ T cells. Thus, the contribution of the activated $CD4^+CD25^+$ T cells to the proliferative responses in the cocultures performed in the presence of IL-2 is also minimal but may contribute some residual proliferation to the culture. The hypothesis (9) that IL-2 abrogates suppressor function is not supported by our studies on $CD8^+$ responders because suppression is clearly maintained in the presence of IL-2.

We next examined the expression of CD25 on F5 $CD8^+$ T cells stimulated either in the presence or absence of $CD4^+CD25^+$ T cells. Following 6 h of stimulation with peptide-pulsed APC, up-regulation of CD25 expression on the F5 $CD8^+$ T cell responders was similar in presence or absence of $CD4^+CD25^+$ T cells (Fig. 3*A*). However, no further up-regulation of CD25 on the $CD8^+$ responders was seen in the $CD4^+CD25^+/CD8^+$ cocultures (Fig. 3*A*). The expression of CD69 was identical on $CD8^+$ responders stimulated in the presence or absence of $CD4^+CD25^+$ cells at both time points tested (Fig. 3*A*). In multiple ($n = 5$) experiments, addition of activated $CD4^+CD25^+$ T cells resulted in $73 \pm 5\%$ suppression of the induction of CD25 when the responding $CD8^+$ T cells were assayed for CD25 expression following 24 h of stimulation. The addition of exogenous IL-2 (100 U/ml) also failed to restore the level of CD25 expression on suppressed responders to levels seen on responders cultured alone (Fig. 3*B*) or responders cultured with activated $CD4^+CD25^-$ T cells (data not shown).

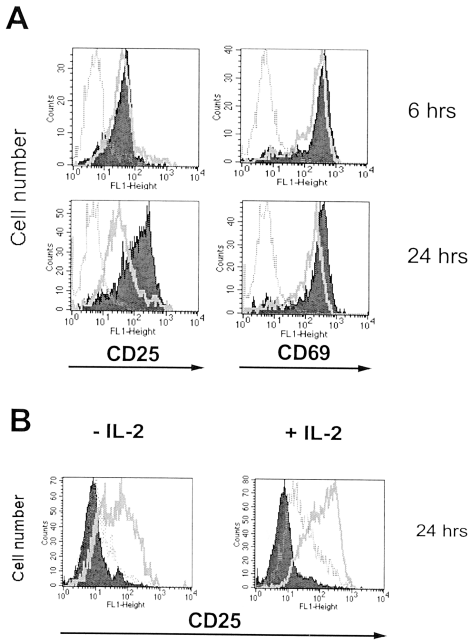

FIGURE 3. $CD4^+CD25^+$ regulatory T cells suppress IL-2Rα expression on responding $CD8^+$ T cells, and IL-2 fails to rescue its expression. *A*, Tg F5 $CD8^+$ T cells (5×10^5) were stimulated with $NP_{366-374}$ peptide-pulsed TdS (2×10^6) either alone (filled line) or with activated $CD4^+CD25^+$ T cells (5×10^5; solid line). FACS analysis for CD25 and CD69 was performed at 6 and 24 h and compared with unstimulated $CD8^+$ T cells (dotted line). *B*, Tg P14 $CD8^+$ T cells (5×10^5) were stimulated with LCMV gp33–41 peptide-pulsed TdS (2×10^6) either alone (solid line) or with activated $CD4^+CD25^+$ T cells (5×10^5; dotted line) in the presence of exogenous IL-2 (100 U/ml). FACS analysis for CD25 was performed at 24 h and compared with unstimulated $CD8^+$ T cells (filled line). All histograms were gated on $CD8^+$ T cells. $CD8^+$ T cells stimulated in the presence of activated $CD4^+CD25^-$ cells displayed CD25 and CD69 expression levels indistinguishable to those observed when $CD8^+$ T cells were cultured alone (data not shown).

Soluble tetramer stimulation of OT-I $CD8^+$ T cells resulted in robust T cell proliferation (Fig. 4*A*) and IFN-γ production (Fig. 4*B*), confirming previous studies (15–17) showing that $CD8^+$ T cells can be efficiently activated in the absence of APC and APC-derived costimulatory signals. $CD8^+$ T cells from normal C57BL/6 mice did not proliferate at any tetramer concentration (Fig. 4*A*), nor did an irrelevant soluble tetramer (H-$2D^b$/CEA antigenic peptide) preparation induce proliferation (data not shown) or IFN-γ production (Fig. 4*B*). To directly assess whether $CD4^+CD25^+$ T cells suppress $CD8^+$ T cell responders by modulating APC function or by direct T-T contact, we stimulated highly purified OT-I $CD8^+$ T cells (>99% pure) with soluble MHC I H-$2K^b$-$OVA_{257-264}$ tetramers, in the presence or absence of titrated numbers of $CD4^+CD25^+$ T cells. Marked suppression of both proliferation and IFN-γ production was seen in the presence of $CD4^+CD25^+$ T cells, but not $CD4^+CD25^-$ cells, even at low $CD4^+CD25^+$:$CD8^+$ ratios (Fig. 4, *C* and *D*). Taken together, the results from this two-cell system conclusively demonstrate that $CD4^+CD25^+$-mediated suppression occurs via a T-T cell interaction, and the APC is not directly required for the delivery of the suppressive signal to responding $CD8^+$ T cells.

$CD8^+$ T cells may contribute to the immunopathogenesis of many autoimmune diseases (18–21). Therefore, it is desirable that regulatory T cells be able to control autoreactive $CD8^+$ T cells as well as $CD4^+$ T cells. Most of the effects of regulatory $CD4^+CD25^+$ T cells on $CD8^+$ responders were similar to those seen with $CD4^+$ responders. However, several important differences should be noted. First, in addition to T cell proliferation, the capacity of fresh $CD8^+$ T cells to manifest effector function such as the production of IFN-γ was also suppressed. Second, whereas $CD4^+CD25^+$ T cells inhibit the activation of $CD4^+$ responders by primarily blocking IL-2 production, $CD4^+CD25^+$ T cells regulate

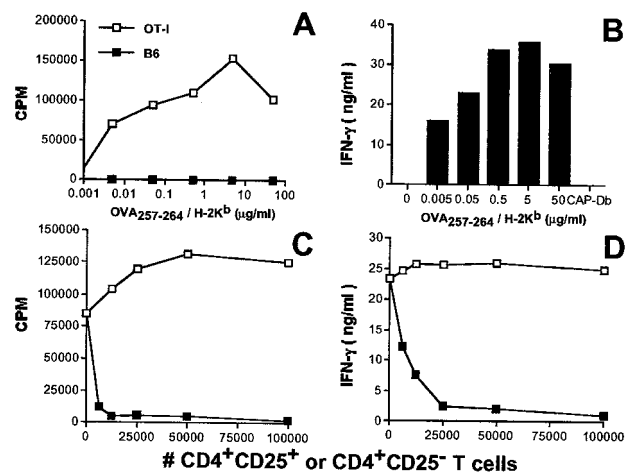

FIGURE 4. Suppression mediated by $CD4^+CD25^+$ T cells is APC-independent. *A*, Tg (OT-I) and non-Tg (wild-type B6) $CD8^+$ T cells (5×10^4) were stimulated with varying concentrations of soluble MHC I H-$2K^b$-$OVA_{257-264}$ tetramers. Proliferation was measured as in Fig. 1. *B*, Culture supernatants were harvested at 48 h, and IFN-γ ELISA was performed. *C*, Tg OT-I $CD8^+$ T cells (5×10^4) were stimulated with soluble MHC I H-$2K^b$-$OVA_{257-264}$ tetramers (0.5 μg/ml) and titrated numbers of activated $CD4^+CD25^+$ (■) or $CD4^+CD25^-$ (□) T cells, and proliferation was assayed. *D*, Supernatants were collected at 72 h, and IFN-γ ELISA was performed.

$CD8^+$ T cell responses both by blocking IL-2 production as well as by lowering responsiveness to exogenous IL-2 and thereby potentially disrupting CD4 help for $CD8^+$ T cells. Finally, $CD4^+CD25^+$ T cells can inhibit T cell activation by directly acting on responder $CD8^+$ T cells in the absence of APC. However, this result does not exclude the possibility that $CD4^+CD25^+$ T cells might also exert inhibitory/deactivating effects on APC or use the APC surface as a platform on which the suppressor cells physically interact with $CD4^+$ or $CD8^+$ effectors in vivo.

Although immunoregulatory $CD4^+CD25^+$ cells function beneficially in vivo to protect the host against the development of autoimmunity, they may simultaneously prevent the host from mounting an immune response to autoantigens such as tumor Ags. Because IL-2 responsiveness by $CD8^+$ T cells is a critical factor for cytokine production (IFN-γ) and cytolytic activity (22, 23), our demonstration that $CD4^+CD25^+$ T cells down-regulate both IL-2 production and CD25 expression on $CD8^+$ T cells may represent a significant impediment to the use of tumor or viral vaccines. Indeed, deletion of $CD4^+CD25^+$ T cells before the use of such vaccines may be needed for optimal immunotherapy.

References

1. Shevach, E. M. 2000. Suppressor T cells: rebirth, function and homeostasis. *Curr. Biol. 10:R572.*
2. Fowell, D., and D. Mason. 1993. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes: characterization of the $CD4^+$ T cell subset that inhibits this autoimmune potential. *J. Exp. Med. 177:627.*
3. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell 101:455.*
4. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol. 155:1151.*
5. Powrie, F., M. W. Leach, S. Mauze, L. B. Caddle, and R. L. Coffman. 1993. Phenotypically distinct subsets of $CD4^+$ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int. Immunol. 5:1461.*
6. Sakaguchi, S., M. Toda, M. Asano, M. Itoh, S. S. Morse, and N. Sakaguchi. 1996. T cell-mediated maintenance of natural self-tolerance: its breakdown as a possible cause of various autoimmune diseases. *J. Autoimmun. 9:211.*
7. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med. 184:387.*
8. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431.*
9. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol. 10:1969.*
10. Thornton, A. M., and E. M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med. 188:287.*
11. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med. 192:303.*
12. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J. Immunol. 164: 183.*
13. Cederbom, L., H. Hall, and F. Ivars. 2000. $CD4^+CD25^+$ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur. J. Immunol. 30:1538.*
14. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol. 162:5317.*
15. Wang, B., R. Maile, R. Greenwood, E. J. Collins, and J. A. Frelinger. 2000. Naive $CD8^+$ T cells do not require costimulation for proliferation and differentiation into cytotoxic effector cells. *J. Immunol. 164:1216.*
16. Lu, P., Y. L. Wang, and P. S. Linsley. 1997. Regulation of self-tolerance by CD80/CD86 interactions. *Curr. Opin. Immunol. 9:858.*
17. Sepulveda, H., A. Cerwenka, T. Morgan, and R. W. Dutton. 1999. CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. *J. Immunol. 163:1133.*
18. Vizler, C., N. Bercovici, A. Cornet, C. Cambouris, and R. S. Liblau. 1999. Role of autoreactive $CD8^+$ T cells in organ-specific autoimmune diseases: insight from transgenic mouse models. *Immunol. Rev. 169:81.*
19. Herrera, P. L., D. M. Harlan, and P. Vassalli. 2000. A mouse CD8 T cell-mediated acute autoimmune diabetes independent of the perforin and Fas cytotoxic pathways: possible role of membrane TNF. *Proc. Natl. Acad. Sci. USA 97:279.*
20. Wong, F. S., I. Visintin, L. Wen, R. A. Flavell, and C. A. Janeway. 1996. CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells. *J. Exp. Med. 183:67.*
21. Wong, F. S., and C. A. Janeway. 1997. The role of CD4 and CD8 T cells in type I diabetes in the NOD mouse. *Res. Immunol. 148:327.*
22. Sad, S., and L. Krishnan. 1999. Cytokine deprivation of naive $CD8^+$ T cells promotes minimal cell cycling but maximal cytokine synthesis and autonomous proliferation subsequently: a mechanism of self-regulation. *J. Immunol. 163:2443.*
23. Mosmann, T. R., S. Sad, L. Krishnan, T. G. Wegmann, L. J. Guilbert, and M. Belosevic. 1995. Differentiation of subsets of $CD4^+$ and $CD8^+$ T cells. *Ciba Found. Symp. 195:42.*