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Antigen Nonspecific Suppression of T Cell Responses by Activated Stimulation-Refractory CD4\(^+\) T Cells\(^1\)

Christine T. Duthoit,* Phuong Nguyen,* and Terrence L. Geiger\(^2\)\(^*\)\(^\dagger\)

Several classes of anergic T cells are capable of suppressing naive T cell proliferation and thereby limiting immune responses. Activated T cells, although not anergic, are transiently refractory to restimulation with Ag. We examine in this study whether activated refractory murine T cells can also suppress naive T cell responses. We find that they can, and that they exhibit many of the suppressive properties of anergic T cells. The activated cells strongly diminish Ag-mediated T cell proliferation, an activity that correlates with their refractory period. Suppression is independent of APC numbers and requires cell contact or proximity. Naive T cells stimulated in the presence of activated refractory cells up-regulate CD25 and CD69, but fail to produce IL-2, and thus to display the suppressive properties of anergic T cells.

R
ecent studies of anergic T cells have shown that they are not only refractory to antigenic stimulation themselves, but are capable of suppressing the proliferative response of naive T lymphocytes (1–3). This suppression appears independent of how anergy is induced; it is mediated by T cells rendered anergic by stimulation with Ag in the absence of costimulation, by T cells rendered anergic by stimulation with T cell APCs, as well as by an anergic lineage of T cells that coexpresses CD4 and CD25 (4–8). In each case the suppression is not mediated by passive delivery of 18 U.S.C. Section 1734 solely to indicate this fact. It is unknown whether the state of refractoriness is intrinsic to the activated CD4\(^+\) T cells, or whether these cells can additionally act in trans, suppressing responses by nonrefractory cells. In this study, we have examined whether stimulation refractory CD4\(^+\) T cells, like anergic T cells, can suppress the proliferative response of naive CD4\(^+\) T cells. We show that they can, and that these activated T cells exhibit many of the suppressive properties previously described in anergic T cells. These transient suppressors have not been previously described and may play a role in limiting immune responses.

Materials and Methods

Animals

AND mice, transgenic for a rearranged pigeon cytochrome \(c\) (PCC)-specific TCR, were bred >20 generations onto the B10.BR background. 24VAVB transgenic mice (16), transgenic for a rearranged collagen II-specific TCR, were provided by Dr. E. Rosloniec (University of Tennessee, Memphis, TN) and were bred with DBA/1 mice for >15 generations. B10.BR and DBA/1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Abbreviations used in this paper: AICD, activation-induced cell death; PCC, pigeon cytochrome \(c\).

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Cells were grown in Eagle’s-Hank’s amino acid (EHAA; BioSource International, Rockville, MD) supplemented with 10% heat-inactivated Premium FCS (BioWhittaker, Walkersville, MD), penicillin G (100 U/ml), streptomycin (100 μg/ml), 292 μg/ml l-glutamine (Life Technologies, Rockville, MD), and 50 μM 2-ME (FisherBiotech, Fair Lawn, NJ). FCS (KAERADLAYLQKATAK) and bovine collagen type II (GEPGIAG FGKEGQFGKGEFG) synthetic peptides were synthesized and HPLC-purified by the St. Jude Children’s Research Hospital Hartwell Center for Biotechnology (Memphis, TN). Con A was purchased from Sigma-Aldrich (St. Louis, MO). FITC-conjugated or biotinylated anti-CD25 (7D4), allophycocyanin-conjugated anti-CD4 (L3T4), PE-conjugated anti-CD69 (H1.2F3), and anti-CD16/CD32 Fc block (2.4G2) were purchased from BD PharMingen (San Diego, CA).

Cell purification

Lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) and spleen were harvested from 6–12 wk old mice. Single cell suspensions were prepared in HBSS by forced passage through a cell strainer, erythrocytes were lysed with Gey’s solution, and T cells purified by incubation on nylon wool columns (Polysciences, Warrington, PA) for 1 h at 37°C in EHAA complete medium. CD4−CD25− cells were purified by staining with Fc block, allophycocyanin anti-CD4, and FITC anti-CD25 Abs in PBS of 5% FCS for 20 min before flow cytometric sorting on a FACStar Cell Sorter (BD Biosciences, San Diego, CA). Sorted cell purity ranged from 97% to 99%.

Cell culture

To produce activated T cells, freshly sorted CD4+CD25− AND T cells were cultured with 3000 rad irradiated B10.BR splenocyte feeders and 500 U/ml recombinant mouse IL-4 (R&D Systems) was also added. No recombinant mouse IL-2 (R&D Systems, Minneapolis, MN). In some cases 500 U/ml recombinant mouse IL-4 (R&D Systems) and anti-CD25 Abs in PBS of 5% FCS for 20 min before flow cytometric sorting on a FACStar Cell Sorter (BD Biosciences, San Diego, CA). Sorted cell purity ranged from 97% to 99%.

Flow cytometry

Cells were stained and analyzed on a FACSCalibur using CellQuest software (BD Biosciences). Cell cycle progression was determined by resuspending the cells at 1 × 10^6 cells/ml in PBS, then adding propidium iodide (0.05 μg/ml) in a 0.1% sodium citrate/0.1% Triton X-100 solution. Samples were incubated 30 min at room temperature in the dark in presence of 0.2 mg/ml RNase before flow cytometric analysis using ModFit LT software (Verity Software, Topsham, ME).

Results

Suppressive activity of stimulation-refractory T cells

To determine whether T cells develop suppressive activity after activation, we stimulated freshly sorted CD4+CD25− AND T cells with irradiated B10.BR feeders, PCC peptide, and recombinant mouse IL-2. The cells were flow cytometrically selected for the absence of CD25 to prevent contamination with CD4+CD25− immunoregulatory T cells that have been previously found to be suppressive (Fig. 1A). High-dose recombinant mouse IL-2 (10 ng/ml) was added as the length of the refractory state is proportional to the dose of IL-2 used (13, 14). Expression of the CD25 high-affinity IL-2R, a cell surface marker for activation, peaked 3–4 days after stimulation, with detectable expression on >90% of cells (Fig. 1B and data not shown). Other indicators of T cell activation, including up-regulation of CD44 and CD69, were likewise apparent. In multiple experiments T cells stimulated for this period and then restimulated with PCC showed a 50% to >95% reduced proliferation compared with equal numbers of similarly stimulated naive T cells. To test whether these refractory T cells were suppressive, day 4-stimulated T cells were flow cytometrically sorted and cocultured at a 1:1 ratio with freshly sorted CD4+CD25− AND T cells and irradiated feeders, either without stimulation, with PCC peptide, or with Con A. The activated T cells failed to respond to Ag themselves (data not shown), and suppressed naive T cell proliferation to Ag by 90.2% and to Con A by 93.8% compared with control T cells stimulated in the absence of activated T cells (Fig. 1C). This demonstrates that restimulated activated T cells are capable of suppressing the proliferation of naive T lymphocytes.

Suppression by activated T cells correlates with activation-induced stimulation refractoriness

We next asked whether suppression by activated T cells correlated with their period of refractoriness. To test for this we stimulated freshly sorted CD4+CD25− AND T cells with PCC peptide and high-dose IL-2 for different times, sorted the activated T cells, and restimulated them with PCC peptide alone or in cocultures with freshly sorted naive CD4+CD25− AND T cells. The naive T cells were also cultured in the absence of activated cells as controls. We measured proliferation at 72 h by [3H]thymidine incorporation.

To define the period of refractoriness among the activated cells, we analyzed the ratio of the preactivated cells’ Ag-induced proliferation with that of simultaneously stimulated cultures of equal numbers of freshly isolated CD4+CD25− AND T cell controls. Proliferation refractoriness began ~2–3 days after primary or secondary stimulation and lasted for several days (Fig. 2A, right). To determine whether the refractory activated T cells suppressed naive T lymphocyte proliferation, we measured the relative Ag-induced proliferation of naive T cell cultures to that of cultures to which a 1:1 ratio of activated T cells were added (Fig. 2A, left). A
A strong correlation was observed between periods of activation-induced nonresponsiveness and the suppressive activity of the stimulated T cells ($p < 0.0001$ at a cutoff naive to activated proliferation ratio of 2.0) (Fig. 2). Interestingly, the suppressive activity and refractory period appeared more rapidly and persisted longer after secondary and subsequent stimulations than after primary stimulation (Fig. 2A and data not shown).

Because the $[^3]$H]thymidine incorporation assays did not distinguish proliferation by the activated T cells and their naive targets, we could not determine whether the naive T cells proliferated at times when the activated cells were fully responsive to Ag or whether they were suppressed then too. To test for this, we labeled the naive T cells with the membrane binding dye CFSE, and as an indicator of cell division, followed their loss of CFSE-staining intensity. Naive T cells cocultured with nonrefractory day 10 activated T cells proliferated comparably to control cocultures in which the CFSE-labeled cells were mixed with equivalent numbers of unlabeled naive T cells (data not shown). These results demonstrate that activated T cells are transiently suppressive after stimulation and that this suppressive activity corresponds to their own stimulation refractoriness.

Considering that the suppressive function of the activated cells peaked at 4 days after activation, in subsequent experiments, described below, we analyzed suppression by activated T cells 4 days after ex vivo stimulation with Ag. All experiments included parallel proliferation studies confirming that the activated T cells were suppressive at the time of study (data not shown).

**Division of naive T cells is suppressed by the presence of activated T cells**

In our initial studies, the activated T cells may have reduced naive T cell proliferation, measured by $[^3]$H]thymidine incorporation, either by inhibiting mitosis or by promoting cell death. To distinguish between these possibilities we directly analyzed proliferation

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**FIGURE 1.** Proliferation suppression by activated T cells. A, Isolation of CD4$^+$CD25$^-$ AND T cells. Plots of CD4 and CD25 stained populations before and after flow cytometric sorting are shown. B, Phenotype of stimulated cells. CD25 and CD69 expression is shown 4 days after sorted CD4$^+$CD25$^-$ AND T cells were stimulated with irradiated feeders, PCC peptide, and IL-2. Unstimulated cells did not show significant expression of these markers (data not shown). C, Freshly sorted CD4$^+$CD25$^-$ naive T cells were cocultured at a 1:1 ratio with the activated T cells or cultured alone without stimulation, or with PCC peptide or Con A. Proliferation was assessed by measuring $[^3]$H]thymidine incorporation at 72 h. Percentage of suppression was calculated as: $100 \times (cpm$ naive cells $- cpm$ coculture)/$cpm$ naive cells. Mean $\pm$ 1 SD of triplicate samples from one of four similar experiments is shown.

**FIGURE 2.** Time course of suppression and activation-induced stimulation refractoriness. A, Freshly isolated CD4$^+$CD25$^-$ AND T cells were stimulated with irradiated feeders, PCC peptide, and IL-2 for the indicated number of days (abscissa) before flow cytometric purification of the activated T cells. CD4$^+$CD25$^-$ naive T cells were then cocultured at a 1:1 ratio with the activated T cells in presence of feeder cells and PCC peptide. CD4$^+$CD25$^-$ naive T cells or activated T cells were also cultured alone in the same conditions as controls. Percentage of suppression was calculated as in Fig. 1 (left axis, $\bullet$). To monitor stimulation refractoriness of the activated T cells, the ratio of proliferation of naive cells and activated cells, identically assayed, was determined (right axis, $\circ$). Activated cells were followed through two cycles of stimulation, as indicated by the arrows. B, Refractoriness of (abscissa) and suppression by (ordinate) activated T cells are directly compared. Data are from that plotted in A. Results are expressed as the mean $\pm$ 1 SD of triplicate samples. One of two similar experiments is shown.
of the naive cell population. We labeled the CD4+CD25− naive T cells with CFSE before coculture with unlabeled day 4-activated T cells (filled profile) or CD4+CD25− naive T cells (open profile) and stimulated as in Fig. 1. Cell division was assessed by CFSE staining intensity at 24, 48, and 72 h of coculture by flow cytometry. B, Percentage of CFSE− T cells (ordinate) compared with cell cycle number (abscissa) from data in A is shown. C, Cell cycle analysis averaged from six independent experiments is shown. Experiments were performed as in A and B. Error bars represent ± 1 SD.

**FIGURE 3.** Division of naive T cells is inhibited by the presence of activated T cells. A, CD4+CD25− naive T cells were CFSE-labeled before coculture with unlabeled day 4-activated T cells (filled profile) or CD4+CD25− naive T cells (open profile) and stimulated as in Fig. 1. Cell division was assessed by CFSE staining intensity at 24, 48, and 72 h of coculture by flow cytometry. B, Percentage of CFSE− T cells (ordinate) compared with cell cycle number (abscissa) from data in A is shown. C, Cell cycle analysis averaged from six independent experiments is shown. Experiments were performed as in A and B. Error bars represent ± 1 SD.

Naive T cells are stimulated in the presence of activated T cells

It was possible that the failure of naive T cells to proliferate in the presence of activated T cells resulted from a failure to recognize cognate Ag, for instance due to competition with the activated cells for binding sites on APC. To test for this we assayed proliferation suppression in the presence of graded doses of APC, naive responder cells, and activated T cells. Increasing the amount of splenocyte feeders to T cells to a ratio as high as 50:1 did not alter the suppressive effect of the activated T cells (Fig. 4 and data not shown). These results imply that the suppression of naive T cell proliferation does not result from passive competition for APC binding sites.

Despite seemingly adequate numbers of APCs, it was still possible that the naive T cells were unable to recognize Ag in the presence of activated T cells. To determine whether the naive cells responded to stimulation, we measured their expression of the CD25 and CD69 activation Ags. Naive T cells were labeled with CFSE and stimulated with PCC peptide in the presence or absence of activated T cells. CD25 and CD69 surface expression was measured on the CFSE− cells 24, 48, and 72 h after stimulation. By 24 h, the CFSE− cocultured cells up-regulated the CD25 and CD69 markers (Fig. 5). Expression only slightly differed from that of control-activated cells and persisted for 72 h of coculture. These results show that the naive T cells recognize cognate Ag in the presence of activated cells, and that this recognition induces partial signaling leading to CD25 and CD69 up-regulation, but not to proliferation.

**Naive T cells are blocked in the G0/G1 phase of the cell cycle**

After antigenic stimulation T cells begin to progress through the cell cycle. To determine where the naive T cells were blocked when stimulated in the presence of preactivated T cells, we labeled them with CFSE before coculture and analyzed their cell cycle progression. As expected, 24 h after stimulation no differences were seen between the CFSE− cells cocultured with activated cells or with control naive T cells (Fig. 6). A total of 87.3% of the cells...
cultured with activated cells and 85.3% of those in the control cultures were in the G0/G1 phase, 12.3% and 14.2%, respectively, progressed to the S phase, and almost no cells were dividing (G2/M phase). At 48 h, however, the control cultures showed good progression to the S (64.7%) and G2/M (5.1%) phases. In contrast, 72.2% of CFSE\textsuperscript{+} H11001 cells cocultured with preactivated cells remained in G0/G1, with only 24.8% in S and 3.0% in G2/M. This demonstrates that the activated T cells prevent S phase progression following TCR stimulation, and suggests that there is an early block in the induction of cell cycling.

Suppression by activated T cells is cell contact-dependent

Proliferation suppression mediated by CD4\textsuperscript{+} H11001 CD25\textsuperscript{+} H11002 immunoregulatory T cells or anergic T cells requires close proximity of the effector and target T cell populations; suppression is not observed when targets and effectors are separated across a permeable barrier. To determine whether suppression by activated T cells has similar cell contact dependence, we separated preactivated and naive T cells using 0.4 \mu m polycarbonate membranes in a Transwell dual chamber culture system, stimulating cells in each chamber with peptide Ag and APCs. The membrane prevents cell contact while allowing diffusion of soluble factors between the two chambers. Control wells showed that the activated cells suppressed naive T cell proliferation when they were both present in a single chamber (Fig. 7). However, when activated and naive T cells were on opposing sides of the membrane, the activated cells failed to reduce proliferation by the naive cells. Further, even if activated
and naive T cells were mixed in one chamber, they failed to inhibit proliferation by isolated naive T cells in the second chamber. These results demonstrate that proximity between the activated and naive T cell populations is essential for proliferation suppression. Cocultures of naive and activated T cells with Ag did not produce detectable soluble agents capable of inhibiting naive T cell proliferation.

**Suppression by activated T cells is not Ag specific or MHC restricted**

The experiments we had performed used a single Ag, PCC, to stimulate the activated and naive T cells. To determine the Ag and MHC dependence of the proliferation suppression, we performed similar assays using mixed systems. We used H-2\(^{b}\)-restricted CD4\(^+\)CD25\(^-\) AND T cells that had been stimulated for 4 days with PCC/high-dose IL-2 as effector cells in the suppression assays. These were purified, washed, and cocultured either with freshly isolated CD4\(^+\)CD25\(^-\) AND T cells or with similarly isolated collagen II-specific, H-2\(^{b}\)-restricted 24VAVB T cells. We activated the AND cocultures with PCC peptide and the 24VAVB cocultures with collagen II peptide (50 μg/ml) in the presence of H-2\(^{b}\) or H-2\(^{d}\) feeder cells, respectively. Alternatively Con A was used to nonspecifically stimulate the cells. The activated AND T cells suppressed both Ag and mitogen-induced proliferation of both AND and 24VAVB responder T cells (Fig. 8). Similar experiments showed that activated T cells derived from nontransgenic B10.BR or TCR-transgenic 24VAVB mice effectively suppressed mitogen or Ag-specific proliferation by naive B10.BR, 24VAVB, and AND responder cells (data not shown). Therefore the suppressive activity of activated T cells is neither Ag specific nor MHC restricted.

**Activated T cells inhibit IL-2 production by responding naive T cells**

IL-2 is a critical mediator of T cell proliferation after activation. The activated T cells may have suppressed naive T cell proliferation by blocking the production of or response to this growth factor. To address this we measured the IL-2 content of cultures containing naive AND T cells, day 4-activated AND T cells, or mixed naive and activated T cells after stimulation with Ag. After primary stimulation of naive T cells alone, IL-2 was detectable in culture supernatant within 24 h and was increased at 48 h (Fig. 9A). In contrast, little IL-2 was detected in restimulated cultures of activated T cells or in cocultures of naive and activated T cells. This suggested that the activated T cells blocked IL-2 production by the naive T cells. Alternatively the CD25\(^+\)-activated T cells may have consumed the IL-2 present, thereby reducing its concentration in the culture medium. To distinguish between these possibilities we CFSE-labeled the naive T cell population before activation with Ag in the presence of activated or control naive T cells. After 48 h, the CFSE\(^{+}\) cells were sorted and the presence of IL-2 mRNA in them was assayed by RT-PCR. As expected, freshly isolated unstimulated naive T cells lacked detectable IL-2 mRNA and naive T cells activated in the presence of additional unlabeled naive T cells produced significant amounts of message (Fig. 9B). In contrast, naive T cells cocultured with activated T cells did not produce detectable IL-2 mRNA. This demonstrates that the naive T cells stimulated in the presence of activated T cells failed to transcribe IL-2 message.

To determine whether the defect in IL-2 production was responsible for the suppressive effects of the activated T cells, we added recombinant mouse IL-2 to naive and activated cell cocultures. The addition of even high doses of IL-2 failed to relieve the suppression (Fig. 9C). This occurred despite good expression of the IL-2R α-chain (Fig. 5) and β-chain (CD122, data not shown), demonstrating that naive T cells stimulated in the presence of activated T cells were not only unable to produce IL-2, but failed to respond to it when present.

![FIGURE 8. Suppression by activated T cells is not Ag specific or MHC restricted. H-2\(^{b}\)-restricted CD4\(^+\)CD25\(^-\) AND T cells were stimulated with irradiated feeders, PCC peptide, and 10 ng/ml recombinant mouse IL-2 for 4 days and purified by flow sorting. CD4\(^+\)CD25\(^-\) naive AND or 24VAVB (collagen II-specific, H-2\(^{b}\)-restricted) T cells were then cocultured at a 1:1 ratio with the activated AND T cells or cultured alone in the presence of PCC peptide (5 μM) or bovine collagen type II peptide (50 μg/ml) and H-2\(^{b}\) or H-2\(^{d}\) feeder cells, respectively. Proliferation was measured by [\(^{3}H\)]thymidine incorporation. Percentage of suppression was calculated as: 100 × (cpm naive cells − cpm coculture)/cpm naive cells. Results are expressed as mean ± 1 SD of triplicate samples from one of three similar experiments. bCII, Bovine collagen II.](http://www.jimmunol.org/)

![FIGURE 9. Activated T cells inhibit IL-2 production by responding naive T cells. A. CD4\(^+\)CD25\(^-\) AND T cells were stimulated for 4 days with PCC and irradiated feeder cells and flow purified. CD4\(^+\)CD25\(^-\) naive AND T cells were then cocultured at a 1:1 ratio with the activated T cells in presence of feeder cells and PCC peptide. The naive or activated T cells were also cultured alone using the same conditions. At 24 and 48 h supernatants were harvested and IL-2 measured. B, CD4\(^+\)CD25\(^-\) naive AND T cells were labeled with CFSE. RNA was isolated from some of these cells (left lane). The remaining cells were cocultured with unlabeled naive or activated AND T cells at a 1:1 ratio for 48 h. CFSE\(^{+}\) cells flow cytometrically sorted, and their RNA extracted. This was reverse transcribed and PCR analyzed for mouse IL-2 and actin as a control. C, CD4\(^+\)CD25\(^-\) T cells were stimulated in the presence or absence of an equal number of day 4-pretactivated T cells with PCC, irradiated splenocyte feeders, and the designated concentration of recombinant mouse IL-2 for 3 days. Proliferation was measured by [\(^{3}H\)]thymidine incorporation. Percentage of suppression was calculated as 100 × (cpm naive cells − cpm coculture)/cpm naive cells. Results in A and C are expressed as mean ± 1 SD of triplicate samples from one representative experiment. One of three similar experiments is shown.](http://www.jimmunol.org/)
Suppressed T cells are not anergic

Naive T cells require only as little as an 8 h exposure with Ag to initiate their proliferative program (17, 18). Beyond this point, continued Ag presence is not required for proliferation. It was possible that the naive T cells stimulated in the presence of activated T cells were incapacitated by the activated cells, but could respond if removed from their presence. To test for this, we labeled freshly isolated CD4⁴ CD25⁻ naive T cells with CFSE and cocultured them with feeder cells and PCC Ag in the presence or absence of activated CD4⁴ CD25⁻ T cells. The CFSE² cells were flow cytometrically purified 24 or 48 h later and recultured in medium. At 24-h intervals aliquots were removed and analyzed for proliferation. T cells cultured in the absence of activated cells, isolated, and recultured in medium alone proliferated well over the following 72 h (Fig. 10A). In contrast, similarly isolated cells from cocultures with activated T cells failed to proliferate. This demonstrates that the continued presence of the activated T cells was not required to prevent proliferation of the responder T cells.

To determine whether the cocultured T cells were anergic or refractory to antigen stimulation themselves, we recultured the isolated CFSE² cells with irradiated APC and PCC peptide in the absence of exogenous cytokine. Antigenic stimulation was capable of inducing proliferation of cells isolated from cultures either with or without activated T cells (Fig. 10B). Cells that had been previously cocultured with activated T cells were delayed in their response relative to control cells, reflecting their ineffective initial stimulation in the presence of the activated suppressive T cells. However, their response was similar to that of freshly isolated naive T cells (Fig. 3C). Further, the naive T cells derived from cocultures with activated cells proliferated similarly to control cells that had been effectively stimulated for the same number of days. Thus T cells that were ineffectively stimulated due to the presence of activated T cells for 24 h, sorted, and cocultured for an additional 2 or 3 day, proliferated equivalently to control T cells effectively stimulated for 24 h, but restimulated for only 1 or 2 day, respectively (Fig. 10, C and D). This shows that T cell proliferation is dependent on the total time of effective stimulation. These results further imply that the large majority of cocultured T lymphocytes are neither anergic nor refractory to stimulation. Implicitly, stimulation in the presence of activated T cells induces a partial activation program that includes some features of normal T cell activation, such as CD25 and CD69 expression, but not others, including IL-2 production and responsiveness. The cells are however capable of full responsiveness if removed from the activated suppressive T cells and restimulated with Ag.

Discussion

Mechanisms that restrain T cell activity are critical to immune function, preventing autoimmunity and down-regulating immune responses. Defining these mechanisms is essential to understanding immune regulation. T cells rendered anergic by activation in the absence of costimulation or by stimulation using T cell APCs, and T cells belonging to the naturally anergic CD4⁴ CD25⁻ T cell lineage have recently been shown to suppress bystander T cell proliferation in vitro and down-modulate autoimmune responses in vivo (1–3). These T cells can therefore restrict the initiation or promulgation of immune responses.

Under normal activation conditions T cells develop a transient stimulation refractoriness (13, 14). The duration of the stimulation-refractory condition is proportional to the strength of the stimulation and quantity of IL-2 present. In this report we have studied whether stimulation-refractory T cells, like anergic T cells, are able to suppress T lymphocyte responses.
Several conclusions can be made. Most importantly we show that activated stimulation refractory T cells are able to suppress Ag and mitogen-specific proliferative responses of naive T cells. This did not result from the contamination of our activated T cells with CD4⁺CD25⁺ immunoregulatory T cells, as flow cytometrically purified CD4⁺CD25⁻ cells were used to generate the activated T cell populations studied. Although the magnitude of the suppression varied between experiments, it generally was nearly complete, with little detectable response to Ag. Suppression did not result from competition for APC binding sites. Addition of splenocyte feeders at up to a 50:1 ratio with T cells did not alter the suppression. More significantly, the responder T cells that failed to proliferate in the presence of activated T cells engaged Ag and were partially stimulated, as they up-regulated both the CD25 and CD69 activation markers. Despite this limited stimulation, the cells failed to enter cell cycle and were blocked at the G₀/G₁ phase. Stimulation in the presence of activated T cells further failed to induce IL-2 transcription and production.

The failure to produce IL-2 in response to Ag was not the exclusive cause of the proliferation defect. Suppression was not affected by the addition of even high levels of IL-2 to the cultures. As high affinity IL-2R was present on the suppressed T cells, this seems not to have resulted from an inability to recognize IL-2. Implicitly, TCR signaling pathways that permit T cell responsiveness to the mitogenic effects of IL-2 were not induced. Other studies likewise demonstrate that IL-2 is inadequate to induce proliferation in some IL-2R⁺ populations, suggesting that this IL-2 unresponsiveness is a shared mechanism restricting T cell response (19, 20).

IL-2 induces a complex signal that can promote proliferation, survival, as well as sensitivity to death receptor signaling (21). These different effects are not integrally linked. In one study, undivided activated T cells that were isolated and stimulated with IL-2 were found to phosphorylate Akt and up-regulate Bcl-2 and Bcl-xL, but failed to proliferate (19). Similarly, treatment of T cells with TGF-β blocks IL-2-induced proliferation, but not the anti-apoptotic effects of IL-2 (22).

Proliferation induction by IL-2 is mediated by Shc- and Stat5-dependent pathways, although Stat5 appears dominant in primary T lymphocytes (23, 24). Our results showing blockade of IL-2-induced proliferation therefore may imply that activated suppressor T lymphocytes induce factors inhibiting effective Stat5 signaling by the IL-2R. Alternatively, TCR stimulation may normally act not only to induce high-affinity IL-2R expression, but to fully enable the IL-2 signal transduction pathway. In the presence of activated-suppressor T cells, high-affinity IL-2R may be induced, but not other components of the signaling pathway necessary for Stat5-mediated induction of cell cycle progression. For example, expression or phosphorylation of Stat5 may be defective, critical Stat5-regulated genes may be inaccessible to this transcription factor, or coactivators of gene expression may not be induced. Indeed, TGF-β-induced inhibition of IL-2 signaling appears to act not on upstream signaling but selectively on target genes such as c-myc and cyclin D₂ (22). Establishing the biochemical basis for IL-2 refractoriness will therefore be essential to understanding the mechanism of activated T cell-mediated suppression.

Continuous presence of the suppressive activated T cells was not responsible for the failure of the naive T cells to respond to Ag. T cells stimulated with Ag for even a few hours receive adequate TCR stimulation to permit proliferation if the cells are then removed from Ag (17, 18, 25). However, T cells cocultured with day 4-activated cells failed to proliferate if removed from the activated cells even 48 h after stimulation with Ag. Implicitly the activated cells did not serve to maintain the presence of a short-lived factor preventing cell cycle progression.

To determine whether the activated suppressor T cells conferred an anergic state on the responding T cells, we sorted the suppressed responders after 24 or 48 h of stimulation in the presence of activated T cells and restimulated them with Ag and APC. The cells were fully responsive to Ag, showing that their limited stimulation with Ag did not induce an anergic or refractory state. Thus the activated T cells prevent naive T cell responses by profoundly restricting their activation, but do not appear to induce long-lasting changes in them.

Proliferation suppression mediated by activated T cells resembles in many aspects that induced by anergic or CD4⁺CD25⁺ T cells. CD4⁺CD25⁺ T cell suppression is not Ag specific or MHC restricted, as is true in our system (26). Likewise some, but not other, models of suppression induced by anergic T cells show a lack of Ag specificity (5, 6). Furthermore, in all cases, suppression requires cell contact or close proximity, as evidenced by Transwell assays, and is therefore seemingly not dependent on down-modulatory soluble biological response modifiers. This suggests a common suppressive mechanism. However, without data demonstrating the molecular basis for such a mechanism, such commonality remains hypothetical. Indeed, both CD4⁺CD25⁺ T cells and anergic T cells have been shown capable of inhibiting APC as well as directly suppressing target T cells, implying that these cells may operate through multiple mechanisms (27–30). It is possible that only some of these mechanisms are shared among the different types of suppressor cells.

Recently, Tham and Mescher (15) studied the refractory state of CD4⁺ T cells stimulated with anti-CD3/B7/ICAM-coated beads. They found that T cells restimulated 3 days after initial activation showed decreased [³H]thymidine incorporation that was reversed by the addition of z-vad or anti-fas ligand. CD4⁺ T cell refractoriness in their system therefore seemed to result from increased AICD upon Ag restimulation. Whether AICD is similarly increased in the suppressive activated T cells used in this study, which were stimulated with Ag-APC, remains to be determined. Viable cell counts of restimulated cultures of activated suppressor cells and naive T cells showed that total cell numbers were maintained, suggesting that significant AICD was not present in our cultures (data not shown). Regardless of its presence, we do not believe that AICD induced by restimulation of the activated CD4⁺ T cells is required to endow them with suppressive properties. Activated AND T cells suppressed naive collagen II-stimulated 24VAVB T cells and vice versa. This shows that the suppressive properties of the activated cells, in contrast to their potential AICD, are independent of their reactivation.

The results of this study support the concept that stimulation refractoriness is a physiologic response of T cells that not only limits responses of the Ag specific T cells themselves, but also limits bystander T cell activation. We speculate that this bystander suppression may apply a brake to immune stimulation within specific milieu, preventing unchecked amplification of the immune response and associated immunopathology. The more intense the stimulation, the greater the number of activated T cells and hence bystander suppression of the ongoing T cell response. Indeed in many in vivo models, continuous high-dose Ag results in a state of immune paralysis, in which T cells become unresponsive to stimulation (31–35). Bystander suppression by activated T cells may provide balance in immune responses so as to prevent immune exhaustion and thereby allow effective memory T cell development (36, 37). Limits on the recruitment and activation of new T cells by activated suppressive T cells may also conceivably help determine the hierarchy of dominance in immune responses by...
influencing T cell competition (38, 39), and may protect against autoimmunity by limiting the stimulatory effects of autoantigens released late in immune responses. Further study will be needed to define the mechanism of action of activated suppressor T cells as well as their full physiologic impact.

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References