The Journal of Immunology

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J Immunol 2008; 181:3323-3335; ;
doi: 10.4049/jimmunol.181.5.3323
http://www.jimmunol.org/content/181/5/3323

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

In Vivo Suppression of Naive CD4 T Cell Responses by IL-2- and Antigen-Stimulated T Lymphocytes in the Absence of APC Competition[1]

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After stimulation, T cells enter a transient refractory period, promoted by IL-2, during which they are resistant to re-stimulation. We previously demonstrated that these IL-2- and Ag-stimulated refractory T cells are able to suppress the Ag-induced proliferation of naive T cells in vitro. We show here that, after adoptive transfer, these T cells are also able to suppress naive T cell proliferation in vivo. More interestingly, potently suppressive T cells can be generated directly in vivo by stimulation with Ag and supplemental IL-2. The activity of the suppressive cells is dose dependent, and the suppressor and suppressed T cells need not be restricted to the same MHC or Ag. Similar to its role in promoting T cell-mediated suppression in vitro, IL-2 is critical for the induction of suppressive activity in activated T cells in vivo. Supplemental IL-2, however, cannot overcome the suppressive activity in target T cells, indicating that suppression is not mediated by competition for this cytokine. Although the activated T cells block naive T cell proliferation, the naive cells do engage Ag and up-regulate the CD25 and CD69 activation markers after stimulation. Therefore, activated T cells stimulated in the presence of IL-2 develop MHC- and Ag-unrestricted suppressive activity. These results provide a new mechanism for competition among CD4$^+$ T lymphocytes, in which initial waves of responding T cells may inhibit subsequently recruited naive T cells. They further suggest a novel negative feedback loop limiting the expansion of T cell responses that may be present during vigorous immune responses or after IL-2 immunotherapy. ***The Journal of Immunology,*** **2008, 181: 3323–3335.**

Ag exposure evokes responses from only a small subset of circulating T cells. The repertoire of responding T cells need not mirror the preimmune Ag-specific T cell repertoire, and can further evolve with time. This frequently leads to an immune response dominated by relatively few T cell clones. One mechanism guiding repertoire evolution is T cell competition for Ag (1). Competition has been most clearly seen among cells recognizing the same epitope (2–6). In both CD4 and CD8 T cell responses, higher affinity epitope-specific cells are more competitive, or fit. During CD8 T cell responses, some reports have also shown competition among cells specific for distinct epitopes, a phenomenon that has been termed cross-competition (7). Ag and APC availability appear to critically influence T cell competition, implying that more fit T cells are those better able to access and be adequately stimulated by limiting quantities of Ag (8, 9).

Mechanisms besides competition for access to Ag and APC may also help shape the responding T cell repertoire. Exposure to Ag does not only induce T cell stimulation. In some T cells it elicits suppressive functions. It has been hypothesized, though not proven, that the suppression of T cells by other T cells acts as a competitive force that influences repertoire development (10). The suppressive properties of T cells may in cases be transient. In 1984, Fitch and colleagues described that T cells develop a period of refractoriness to restimulation after Ag stimulation in vitro (11). This refractory period is enhanced by T cell stimulation in the presence of high concentrations of the promitotic cytokine IL-2 (12). More recently, using culture systems, we showed that refractory Ag and IL-2-stimulated CD4$^+$ T cells also develop a transient ability to potently suppress naive T cell proliferative responses (13, 14). Suppression is cell contact- or proximity-dependent, and the suppressor and target cells need not recognize the same Ag. Although the suppressed cells do not enter cell cycle, they are stimulated by Ag, up-regulating early activation markers. These in vitro findings raised the possibility that activated T cells may also develop suppressive activity in vivo, and compete for immunodominance by suppressing the responses of naive T lymphocytes.

To test this concept, we analyzed whether activated T cells could suppress naive T cell responses in vivo. We demonstrate that in vitro-generated refractory cells that are adoptively transferred into mice modestly inhibit the naive T cell response to Ag. More interestingly, highly suppressive T cells can be generated in vivo by stimulation with Ag and supplemental IL-2. Suppressive activity correlates with CD25 up-regulation and is suppressor cell dose dependent. Interestingly, the suppressive activity of Ag and IL-2-stimulated T cells is neither Ag nor MHC restricted. Nor is it due to the inability of suppressed naive T cells to access APC or Ag. The suppressed T lymphocytes up-regulate the CD25 and CD69 activation markers despite failing to proliferate. These results therefore provide the first direct evidence for CD4$^+$ T cell cross-competition. They further identify a novel form of T cell suppression and a new level at which T cell competition may occur, not among T cells simultaneously stimulated by Ag, but among waves of T cells, wherein T cells activated early during a developing

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Received for publication February 6, 2008. Accepted for publication July 5, 2008.

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[1] This work was supported by National Institutes of Health Grant R01 AI056153 (to T.L.G.) and by the American Lebanese Syrian Associated Charities/St. Jude Children's Research Hospital (to T.L.G., M.S., and H.I.).

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immune response may focus and restrict that response by suppressing responses by subsequent waves of lymphocytes. This form of immune suppression may be relevant after therapeutic administration of IL-2, such as in the treatment of malignancies (15), or in circumstances in which the production of IL-2 or similarly acting cytokines is abundant.

Materials and Methods

Animals

AND mice, transgenic (Tg)[3] for a rearranged pigeon cytochrome *c* (PCC)-specific, H-2 E^k-restricted TCR, were bred >20 generations onto the B10.BR background (16). The 3A9 mice, Tg for a rearranged hen egg lysozyme (HEL)-specific, H-2 A^k-restricted TCR (17) were also bred with B10.BR mice for >10 generations before use. All animal experimentation was performed in accordance with St. Jude Children's Research Hospital Institutional Animal Care and Use Committee requirements.

Media, reagents, and Abs

Cells were grown in Eagle's-Hank's amino acid (BioSource International)/10% heat-inactivated Premium FCS (BioWhittaker), penicillin G (100 U/ml), streptomycin (100 μg/ml), 292 μg/ml L-glutamine (Invitrogen), and 50 μM 2-ME (Fisher Scientific). PCC (KAERADLIAYLKQATAK) and HEL (DGSTDYGILQINSRW) peptides were synthesized and HPLC-purified by the St. Jude Children's Research Hospital Hartwell Center for Biotechnology. Anti-CD25 (7D4), anti-CD4 (L3T4), anti-CD69 (H1.2F3), anti-Vβ3, anti-Vα11, anti-mouse IL-2 (S4B6), anti-glucocorticoid-induced TNF receptor (GITR; clone DTA-1), and anti-CD16/CD32 Fc block (2.4G2) were purchased from BD Pharmingen. Anti-mouse Foxp3 (FJK-16s) was purchased from eBioscience and intracytoplasmic staining was performed per manufacturer's directions.

Cell preparation

Single cell suspensions were prepared from lymph nodes and spleen of 6- to 12-wk-old mice. Erythrocytes were lysed with Gey's solution. For in vitro studies, $CD4^+CD25^-$ T cells were purified by staining with Fc block, anti-CD4, and anti-CD25 Abs in PBS with 5% FCS for 20 min before flow cytometric sorting on a MoFlo high-speed sorter (DakoCytomation). Sorted cell purity ranged from 97 to 99%.

In vitro generation of refractory T lymphocytes

A total of 10×10^6 ml^{-1} flow cytometrically-purified $CD4^+CD25^-$ T cells were stimulated with a 3- to 5-fold excess of irradiated splenocyte feeders, 5 μM PCC peptide, and 100 U ml^{-1} recombinant human (rh) IL-2 (National Cancer Institute Biological Resources Branch Repository) for one or two stimulation cycles, splitting the cells as needed and providing fresh IL-2 containing medium every 3 to 5 days. Cells were washed and analyzed 4 days after stimulation, at which time we previously demonstrated they are highly refractory to restimulation (14).

CFSE proliferation assay

Flow cytometrically-purified $CD4^+CD25^-$ AND T cells or total splenocytes and lymph node cells, after erythrocyte lysis, were washed and resuspended at 10×10^6–50×10^6 cells ml^{-1} in 5 μM CFSE (Molecular Probes)/PBS/5% FCS for 8 min at 37°C. The cells were then washed three times to remove excess CFSE. In some cases, the CFSE-labeled cells were mixed at a 1:1 ratio with unlabeled T cell populations. The cells were stimulated with a 3- to 5-fold excess of 3000 rad-irradiated splenocyte feeders and 5 μM PCC peptide. In the experiments in Fig. 11, 3×10^5 of the designated non-irradiated splenocytes were added to 10^5 naive CFSE-labeled $CD4^+CD25^-$ AND T cells. Cocultures were analyzed by flow cytometry 3 days later or at the designated time using a FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences).

In vivo proliferation assay

Unlabeled RBC-lysed splenocytes containing 10×10^6 or the designated number of AND or 3A9 T cells were administered i.v. (retro-orbital) into B10.BR mice. Recipient mice were then stimulated by administration of 100 μmol PCC or HEL peptides i.v. in 100 μl saline through the alternate retro-orbital plexus. In some experiments, 50,000 IU rhIL-2 was administered i.p. every 12 h for 60 h (6 doses total). After 3 days (~72 h), 10×10^6 CFSE-labeled AND T cells were administered i.v. and PCC and/or HEL peptides subsequently administered. Spleen cells were obtained 24–72 h after peptide administration and analyzed by flow cytometry. CFSE-stained AND cells were identified by additional staining with anti-Vβ3, anti-Vα11, and anti-CD4 Abs as indicated.

Proliferation and cytokine analysis of in vivo treated cells

A total of 10^7 AND T cells were adoptively transferred and left unstimulated or stimulated with Ag and/or IL-2. $V\beta3^+V\alpha11^+$ AND T cells were purified by sorting from splenocytes and stimulated with Ag/APC to assess cytokine production and proliferation. Culture supernatants were assayed at 48 h for cytokines by Bio-Plex (Bio-Rad) according to the manufacturer's protocols. Alternatively, cultures were pulsed with 1 μCi of [^{3}H]TdR after 72 h and harvested ~16 h later on filtermat for scintillation counting (Wallac-LKB).

Results

Refractory T cells suppress naive T cell responses in vitro and in vivo

To confirm that Ag and IL-2-stimulated refractory AND Tg T cells could suppress naive T cell responses in vitro, we cultured them at a 1:1 ratio with freshly isolated, CFSE-labeled $CD4^+CD25^-$ AND T cells. In control cultures, the refractory T cells were replaced with unlabeled $CD4^+CD25^-$ AND T cells. When control cultures lacking refractory T cells were stimulated with PCC peptide, the CFSE-labeled T cells proliferated vigorously, as determined by the progressive dilution of CFSE in the postmitotic cells over 72 h. In contrast, naive T cells cocultured with refractory T cells showed minimal proliferation in response to Ag (Fig. 1, *A* and *B*), demonstrating that they are efficiently suppressed by the refractory cells. We have previously shown that despite their failure to enter cell cycle, naive T cells are stimulated in the presence of the refractory cells, up-regulating the CD25 and CD69 early activation markers (14).

To determine whether the Ag and IL-2-stimulated refractory T cells could similarly suppress T cell proliferation in vivo, we transferred 10^7 of them into unmanipulated syngeneic B10.BR mice. Ten million freshly isolated, CFSE-labeled AND T cells were separately transferred, and the cells were subsequently stimulated by i.v. administration of PCC peptide. After 3 days, the transferred $V_\beta3^+V_\alpha11^+$ cells were analyzed for CFSE fluorescence intensity. Control CFSE-labeled T cells that were not peptide-stimulated showed a single bright CFSE-labeled peak, indicating the absence of proliferation in this population (Fig. 2, *A* and *B*). PCC administration induced significant proliferation. When the prestimulated T cells and naive CFSE-labeled T cells were each transferred, the extent of naive T cell proliferation was diminished, with an ~20% reduction in mean cell cycle number in the experiment shown. Therefore, adoptively transferred stimulation refractory T cells can suppress T cell proliferation in vivo. However, their activity in vivo is substantially less than that seen in vitro.

Suppressive activity of in vivo activated T cells

We were next interested in determining whether similarly suppressive T cells could be generated in vivo. To test this, we transferred 10^7 freshly isolated, unlabeled $CD4^+CD25^-$ AND T cells into B10.BR mice. We stimulated these in a manner resembling their stimulation with soluble antigenic peptide in vitro, administering soluble PCC peptide i.v., with the goal of converting the cells into a refractory and suppressive state. Three days later, freshly isolated, CFSE-labeled $CD4^+CD25^-$ AND T cells were adoptively transferred and PCC peptide was again administered i.v. to the mice. $V_\beta3^+V_\alpha11^+$ cells were analyzed 3 days after the second transfer of cells, and the ability of the preactivated cells to suppress the proliferation of the CFSE-labeled T cells was measured. As in

[3] Abbreviations used in this paper: Tg, transgenic; PCC, pigeon cytochrome *c*; HEL, hen egg lysozyme; rh, recombinant human; Treg, regulatory T cells; GITR, glucocorticoid-induced TNF receptor.

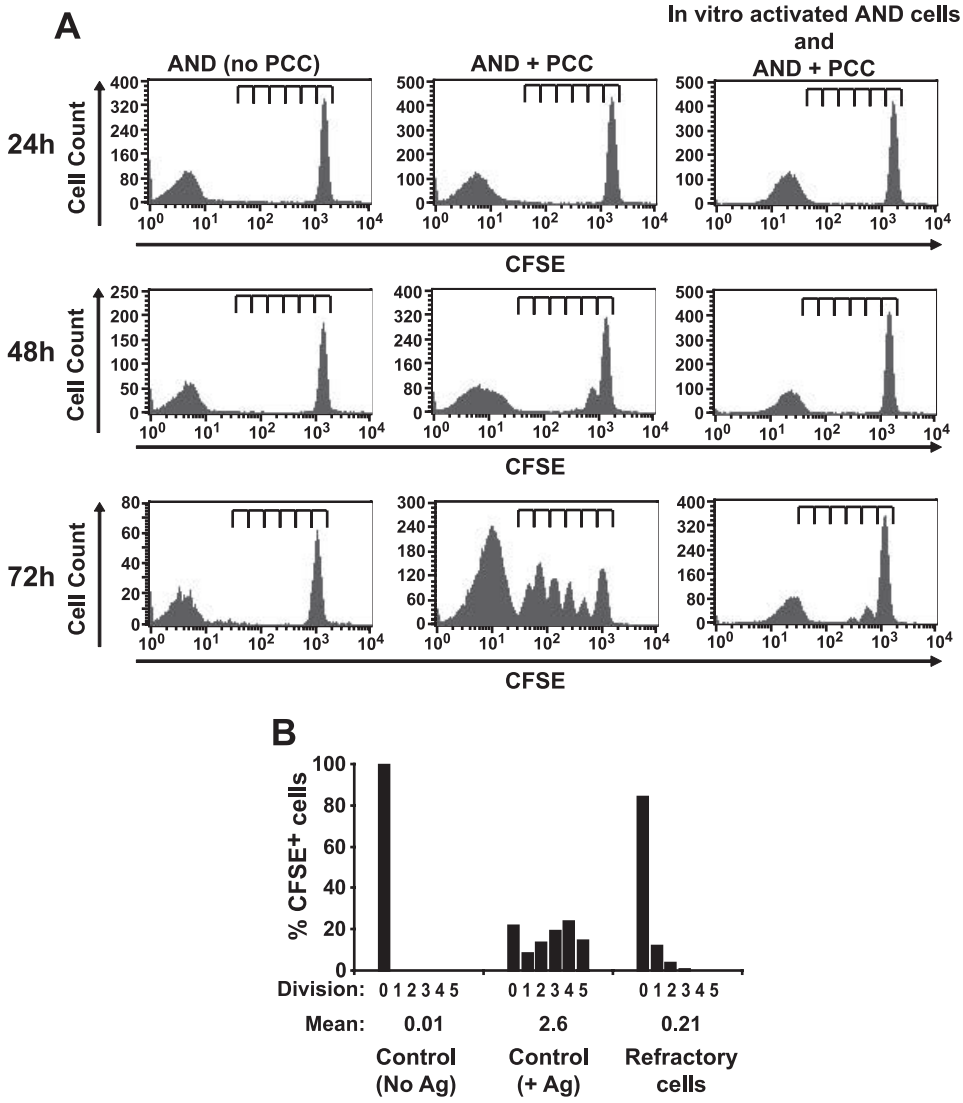

FIGURE 1. Suppression of proliferation by refractory T lymphocytes in vitro. *A*, A total of 5×10^4 CFSE-labeled $CD4^+CD25^-$ AND T cells were stimulated as indicated with irradiated APC in the presence or absence of 5 μM PCC peptide. An equal number of unlabeled $CD4^+CD25^-$ AND T cells (*left* and *center column*) or day 4 activated AND T cells (*right*) were added. Samples were harvested and analyzed by flow cytometry at the indicated times. *B*, Percentage of CFSE-labeled cells at each division cycle from the 72 h data in *A* was calculated based on the flow cytometric peaks.

Fig. 2*A*, CFSE-labeled T cells that were not Ag-stimulated failed to proliferate, whereas control CFSE-labeled cells administered to mice that had not received an initial dose of unlabeled AND T cells proliferated strongly to Ag (Fig. 2*C*, *left* and *left-center panels*). When unlabeled cells were administered and stimulated 3 days before transfer of the CFSE-labeled cells, little suppression of the proliferation of the CFSE-labeled cells was seen, with a small increase in non-cycling T cells apparent in the figure shown (Fig. 2*C*, *center-right panel*). Thus, in contrast to in vitro cocultures, in vivo stimulation with Ag does not induce substantial suppressive activity among the AND T cells.

IL-2 maintains CD25 up-regulation in stimulated AND T cells in vitro and in vivo

IL-2 promotes suppressive activity and refractoriness among Ag-stimulated T cells in vitro (12). Inadequate IL-2 signaling may therefore be one explanation for the limited in vivo suppressive activity we observed with the activated T cells. Activated naive T cells both secrete IL-2 and up-regulate the high affinity IL-2 receptor α-chain, CD25, which is critical for optimal IL-2 signal transduction (18–20). Furthermore, IL-2 maintains and enhances CD25 expression, thereby providing positive feedback for its own signaling (21). Considering the crucial role of CD25 in IL-2 signaling and the persistence and increased expression of CD25 as an indicator of IL-2 signaling, we compared CD25 expression after in vitro and in vivo stimulation.

In vitro, purified CFSE-labeled $CD4^+CD25^-$ AND T cells stimulated with PCC peptide up-regulated CD25 within 24 h of activation (Fig. 3*A*). Levels of CD25 increased progressively over 3 days of stimulation as the cells began to proliferate. To determine the role of IL-2 in this expression, we similarly stimulated the cells in the presence of neutralizing anti-IL-2 Ab. In this circumstance, CD25 expression increased to similar levels as controls at 24 h (Fig. 3*B*). However, CD25 expression did not continue to increase beyond those levels, and began to diminish after the 48 h time point. As has been previously observed, inhibition of IL-2 signaling with neutralizing Ab did not prominently affect proliferation at this early time point (22). Therefore, in vitro, IL-2 is critical for sustaining the early up-regulation of CD25 in peptide-stimulated AND T cells.

We next evaluated the expression of CD25 on freshly isolated, CFSE-labeled AND T cells that were adoptively transferred and activated with i.v. administered PCC peptide (Fig. 4*A*). When cells were stimulated in vivo, CD25 was significantly up-regulated at 24 h. However, this was not sustained, and by 48 h expression had returned to baseline levels. Despite the loss of CD25, the cells continued to proliferate between the 48 h and 72 h time points. Thus CD25 is up-regulated only transiently after T cell activation

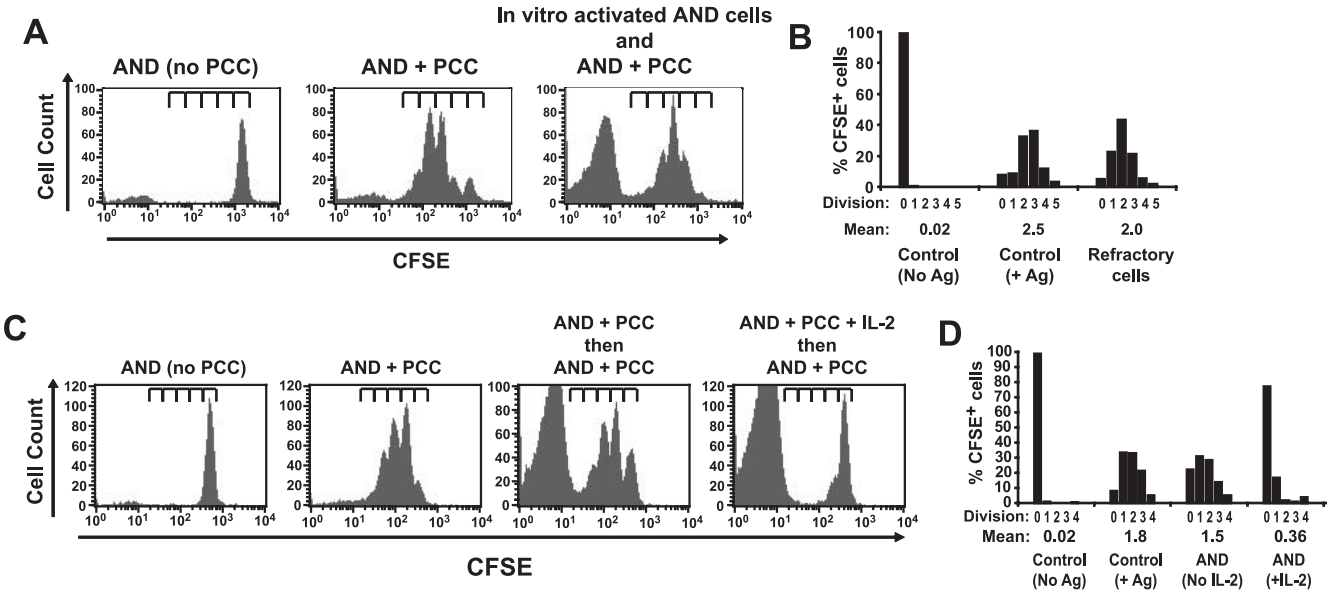

FIGURE 2. Suppression of naive T cell responses by activated T lymphocytes. *A*, A total of 10^7 freshly isolated CFSE-labeled AND T cells were administered with or without an equal number of unlabeled activated T lymphocytes and stimulated with PCC as indicated. Flow cytometrically gated $V\beta3^+V\alpha11^+$ splenocytes were analyzed 3 days later for CFSE expression. *B*, Percentage of CFSE labeled cells at each division cycle from the data in *A* is plotted. *C*, CFSE-labeled AND T cells were transferred and stimulated with PCC peptide as indicated. In some panels, mice were pretreated 3 days prior with unlabeled AND T cells stimulated with PCC peptide with or without rhIL-2. *D*, Percentage of CFSE-labeled cells at each division peak in *C*.

by i.v. Ag in vivo, resembling the CD25 up-regulation pattern in vitro after stimulation in the setting of IL-2 blockade.

In vitro, IL-2 will accumulate in the medium within a culture well. In vivo, the continuous flow of tissue fluid may limit the accumulation of IL-2 at the site of T cell activation. It was thus possible that after stimulation in vivo, insufficient levels of IL-2 and/or similarly acting cytokines were present to sustain CD25 expression. We therefore tested whether increasing T cell exposure to IL-2 may support the retention of CD25 expression in vivo. Administration of twice daily doses of rhIL-2 into mice in which adoptively transferred AND T cells were stimulated with PCC peptide indeed boosted CD25 expression, and the increased CD25 was maintained to the 72 h time point. The impact of IL-2 was specific for CD25. Up-regulation of a second

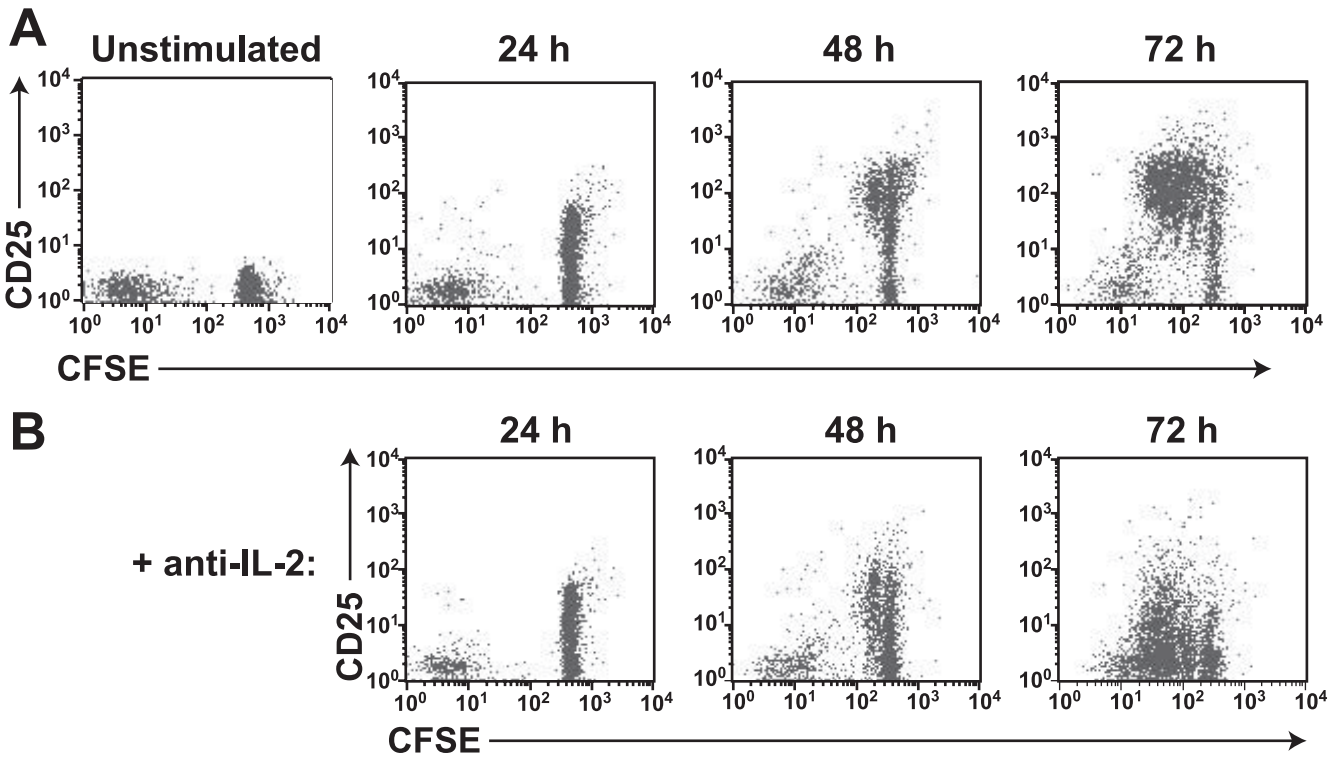

FIGURE 3. CD25 up-regulation on in vitro stimulated AND T cells. *A*, CFSE-labeled $CD4^+CD25^-$ AND T cells were stimulated with PCC peptide and irradiated APC for the indicated times, then analyzed for CD25 expression and CFSE fluorescence by flow cytometry. *B*, Cells were stimulated as in *A* except in the presence of neutralizing anti-IL-2 Ab.

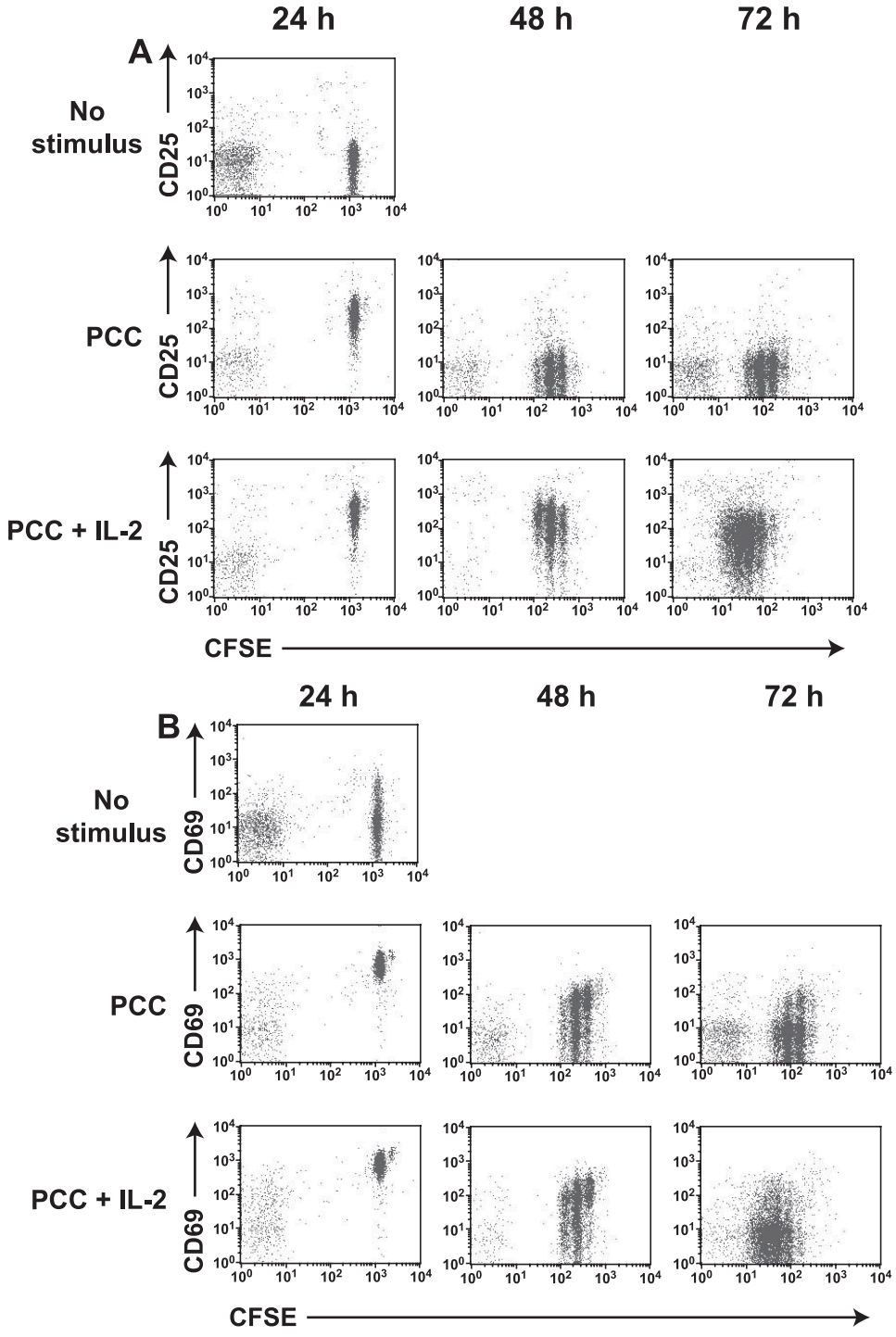

FIGURE 4. CD25 and CD69 upregulation on in vivo stimulated AND T cells. A total of 10^7 CFSE-labeled AND T cells were adoptively transferred and left unstimulated or stimulated with Ag with or without IL-2. $CD4^+V\beta3^+$ gated splenocytes were analyzed 24, 48, and 72 h later for CFSE as well as CD25 (*A*) or CD69 (*B*).

activation marker, CD69, was not influenced by IL-2 treatment (Fig. 4*B*).

Suppression by Ag/IL-2 stimulated T cells in vivo

Considering the suppressive activity of the Ag and IL-2-stimulated $CD25^+$ refractory cells that develop in vitro and the impact of IL-2 on T cell activation in vivo, we were next interested whether the $CD25^+$ T cells generated by Ag and IL-2 stimulation in vivo would also be suppressive. To test this, we adoptively transferred unlabeled AND T cells into syngeneic recipients. These were stimulated with Ag and IL-2 to generate activated $CD25^+$ T cells. We transferred CFSE-labeled AND T cells 3 days later and again administered i.v. PCC peptide. In contrast to the pretransferred cells stimulated in the absence of supplemental IL-2, cells stimulated with extra IL-2 were able to almost completely suppress the naive T cell response (Fig. 2, *C*, *right panel* and *D*). Therefore, similar to our in vitro findings with Ag and IL-2-stimulated refractory T lymphocytes, the proliferation of naive T cells stimulated in vivo in the presence of IL-2- and Ag-activated T cells is suppressed.

In vivo suppression does not result from Ag competition

One possible explanation for the observed suppressive activity is competition for Ag between the initially transferred, IL-2/Ag-stimulated PCC-specific T cells and the subsequently administered naive PCC-specific T cells. This seemed unlikely. AND T cells stimulated in the absence of supplemental IL-2 proliferated strongly,

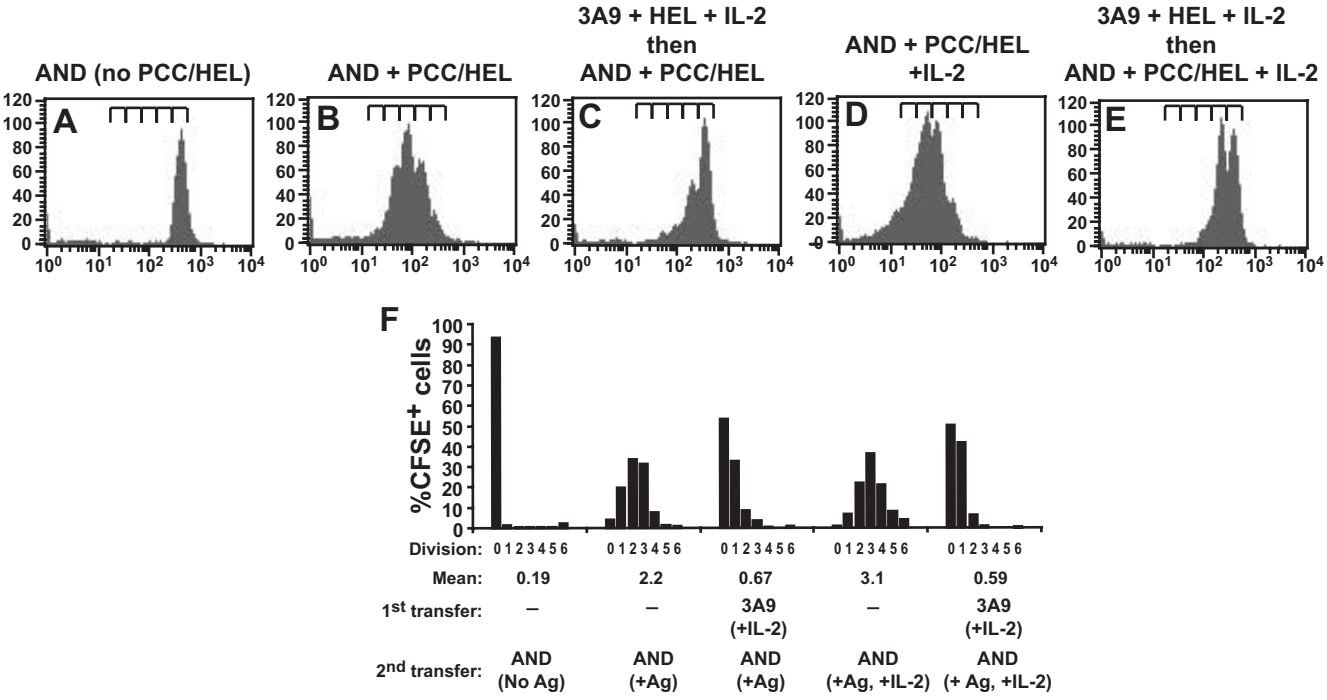

FIGURE 5. Suppression is not Ag-specific or MHC-restricted. *A*, A total of 10^7 CFSE-labeled AND T cells were transferred into syngeneic mice and $V\beta3^+V\alpha11^+$ splenocytes analyzed 3 days later. *B*, PCC and HEL peptides were administered at the time CFSE-labeled AND T cells were transferred. *C*, Three days before treatment as in *B*, 10^7 unlabeled 3A9 T cells were administered and stimulated with HEL peptide and rhIL-2. *D*, The CFSE-labeled AND T cells were stimulated with PCC/HEL and IL-2. *E*, Performed as in *C* except rhIL-2 treatment was continued after administration of the CFSE-labeled AND T cells until analysis. *F*, Percentage of CFSE-labeled cells at each division peak was calculated from the flow cytometry plots in *A–E*.

but did not suppress proliferation by subsequently transferred AND T cells. Nevertheless, it remained possible that the IL-2-stimulated T cells were more efficient in occupying Ag/MHC complexes on APCs, thereby blocking recognition by the subsequently transferred naive T lymphocytes.

To test for this, we analyzed whether T cells specific for an unrelated Ag with a distinct MHC restriction could similarly suppress the naive AND T cell response. Whereas AND Tg T cells are specific for PCC peptide in the context of H-2 E^k, 3A9 Tg T cells recognize a HEL peptide in the context of H-2 A^k. We bred 3A9 TCR Tg mice onto the B10.BR background, then tested whether their T cells could suppress AND T cell responses. The 3A9 T cells were transferred into a B10.BR recipient and stimulated by administering HEL peptide with or without IL-2. We transferred freshly isolated, CFSE-labeled AND T cells 3 days later. We then stimulated both the naive and activated T cells by coadministering HEL and PCC peptide. As with the stimulated PCC-specific cells, when the HEL-specific cells were initially stimulated in the

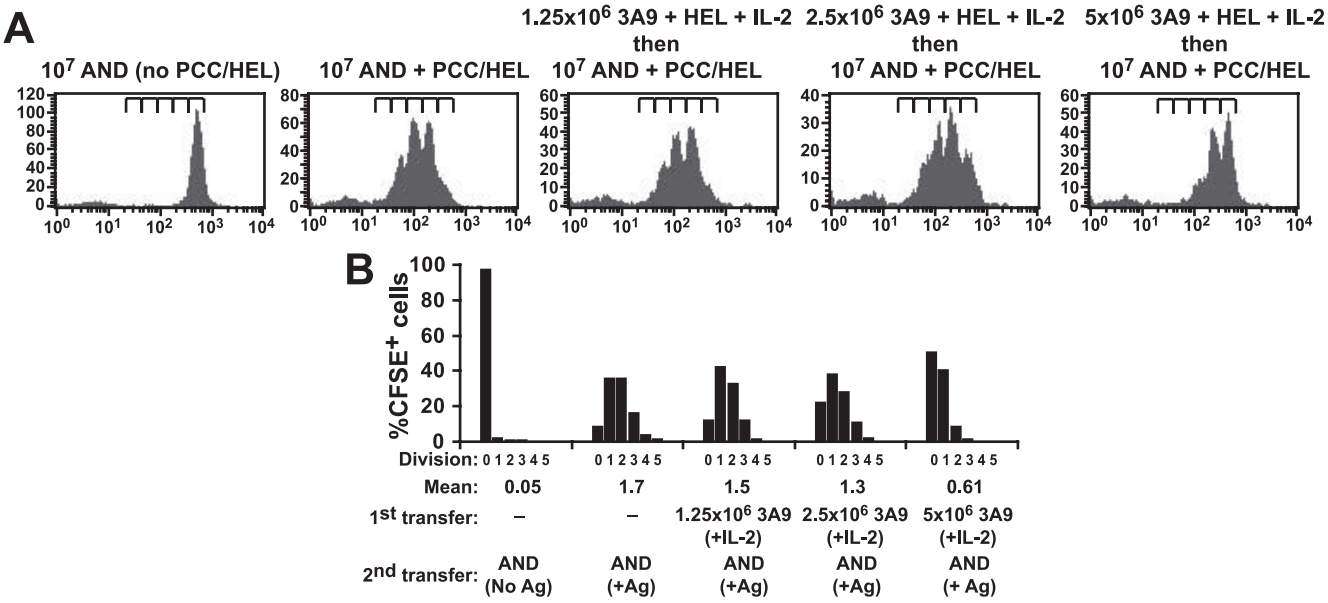

FIGURE 6. Titration of suppression with T cell dose. Analyses were performed as in Fig. 5, except variable doses of 3A9 T cells were administered as indicated. Representative plots are shown in *A*, and percentage of CFSE-labeled cells in each division peak is shown in *B*.

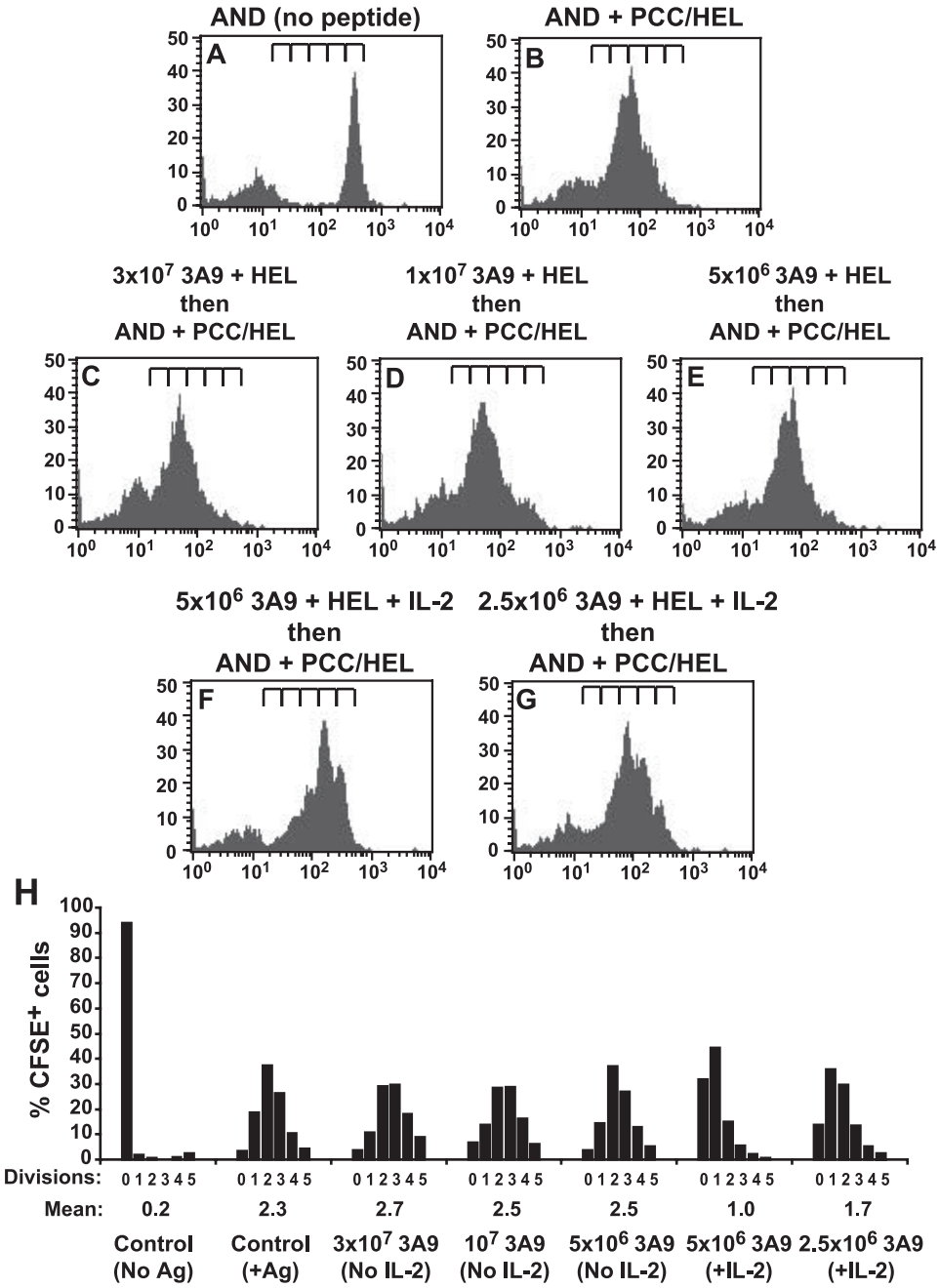

FIGURE 7. Suppression does not result from numeric competition. *A–G*, Analyses were performed as in Fig. 5, except variable doses of 3A9 T cells were administered and stimulated with i.v. Ag with or without IL-2 3 days before administration of a constant dose of 10^7 CFSE-labeled AND T cells. *H*, Percentage of CFSE-labeled cells at each division peak was calculated from the flow cytometry plots in *A–G*.

absence of supplemental IL-2 they failed to suppress the PCC-specific T cell response (data not shown and Fig. 7). In contrast, when supplemental IL-2 was administered when the HEL-specific cells were initially stimulated, the subsequent PCC-specific response was suppressed (Fig. 5, *A–C*, *F*). Therefore, suppression does not result from direct Ag competition among T cells; the suppressor and suppressed cells may be directed against disparate MHC and Ag complexes.

IL-2 supplementation during T cell responses cannot overcome suppression

In some but not other in vitro studies, IL-2 can overcome suppression mediated by Foxp3$^+$ regulatory T cells (Treg) (23–26). To determine whether IL-2 could similarly circumvent proliferation suppression here, we treated mice with supplemental IL-2 both after initial stimulation of the HEL-specific 3A9 T cells, to induce suppressive activity, and after the subsequently administered CFSE-labeled PCC-specific responder T cells were stimulated with Ag. In controls in which the 3A9 T cells were not transferred, CFSE-labeled AND T cells stimulated with PCC and IL-2 underwent more replication cycles than when they were stimulated with PCC alone (Fig. 5, *B*, *D*, and *F*). When an identical stimulation regimen was used in mice pretreated with 3A9 T cells, HEL peptide, and IL-2, the AND T cells responded only weakly to PCC and IL-2 stimulation (Fig. 5, *E* and *F*). Therefore, supplemental IL-2 is unable to overcome the suppression of the naive T lymphocytes.

Titration of suppression with transferred T cell dose

We hypothesized that the suppression in the experiments above was mediated by the pretransferred T cells, and as such should be T cell dose dependent. To test this, we titrated the administered dose of 3A9 T cells. In Figs. 2 and 5, 10^7 T cells were administered both initially and 3 days later. We tested the potency of lower initial doses of 5×10^6, 2.5×10^6, and 1.25×10^6 3A9 T cells,

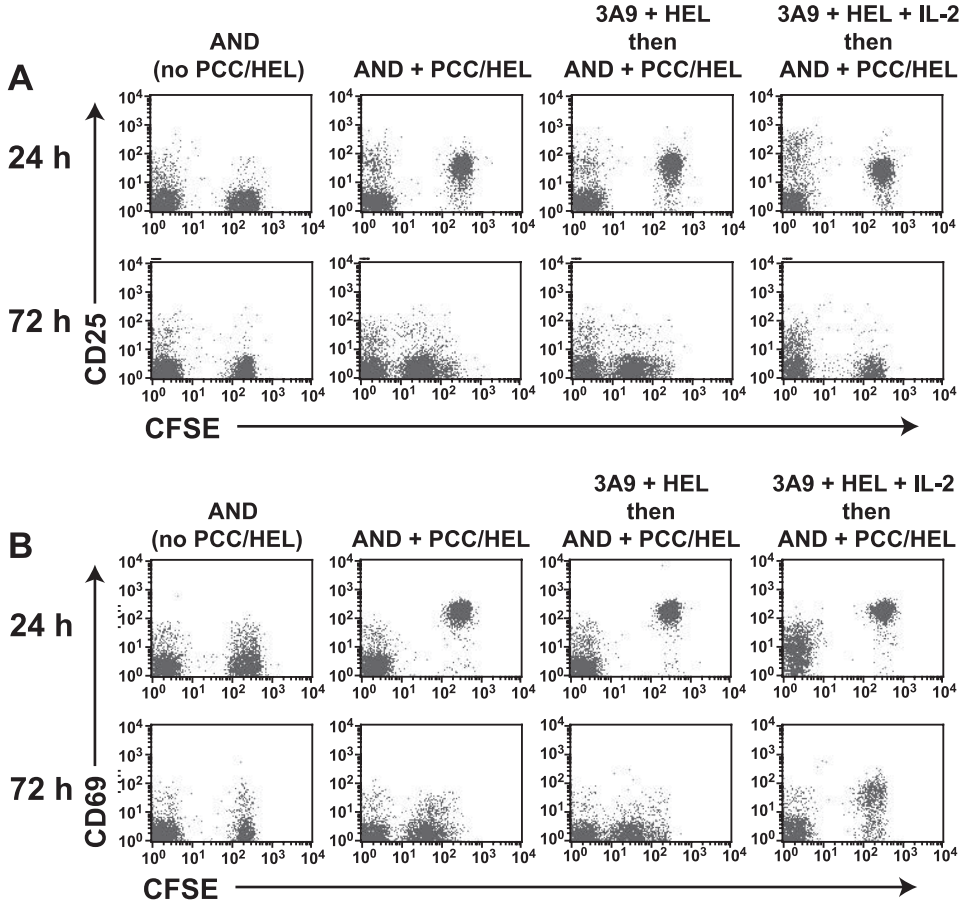

FIGURE 8. CD25 and CD69 up-regulation by suppressed T lymphocytes. A total of 10^7 CFSE-labeled AND T cells were adoptively transferred and left unstimulated or stimulated with Ag. An equal number of 3A9 T cells were adoptively transferred 3 days prior and stimulated with i.v. HEL peptide with or without IL-2. Splenocytes were analyzed 24 and 72 h later. $CD4^+V\beta3^+$ lymphocytes were gated and analyzed for CFSE as well as CD25 (*A*) or CD69 (*B*).

stimulating them with Ag and IL-2 before administering 10^7 CFSE-labeled AND T cells (Fig. 6). At a dose of 5×10^6 3A9 T cells, strong proliferation suppression was observed. At lower doses, however, diminished suppression was seen, with minimal suppression apparent at a dose of 1.25×10^6 T cells. Therefore, suppression is T cell dose dependent. IL-2 and Ag treatment by itself is unable to substantially modify proliferation by subsequently administered and stimulated T lymphocytes in the absence of adequate numbers of activated, suppressive T cells.

Suppression does not result from numeric competition for APC

Analysis of spleens 72 h after adoptive transfer and stimulation of T cells with peptide and IL-2 demonstrated a moderate (up to ~4 times) increase in the number of responding cells when compared with stimulation in the absence of added IL-2 (see for example unlabeled cell peak in Fig. 2*C*, and CFSE-labeled cells in Fig. 4). One possible explanation for the effect of IL-2 was therefore numeric. Enhanced expansion of Ag-specific T cells in the presence of IL-2 may allow Ag and IL-2 stimulated T cells to more effectively compete for APC space, excluding subsequently administered naive T cells. To examine this, we directly compared the suppressive activity of peptide and IL-2 stimulated 3A9 T cells with greater numbers of 3A9 T cells stimulated with peptide in the absence of IL-2.

Doses of $5 \times 10^6 - 3 \times 10^7$ 3A9 T cells were adoptively transferred and stimulated with HEL peptide in the absence of supplemental IL-2. Three days later 10^7 AND T cells were transferred and the cells were stimulated with PCC and HEL peptides. The HEL-specific T cells were wholly unable to suppress the AND T cells regardless of administered dose (Fig. 7, *A–E*, *H*). Different results were apparent when 3A9 T cells (2.5 or 5×10^6) were transferred and stimulated with HEL and IL-2 during the initial 72 h period (Fig. 7, *F* and *G*). Whereas a dose of 3×10^7 cells stimulated without IL-2 did not suppress (Fig. 7*C*), a dose of only 2.5×10^6 cells stimulated with IL-2 was suppressive (Fig. 7, *G* and *H*). As in Fig. 6, the suppression seen after peptide/IL-2 stimulation was dose dependent, with weaker suppression after administering 2.5×10^6 than 5×10^6 3A9 cells. Therefore, the presence of IL-2 during stimulation endows stimulated cells with regulatory activity. The suppressive effects of IL-2 are not secondary to increased expansion of the activated T cells.

Suppressed T cells recognize and are stimulated by Ag

Although numerical competition for APCs was not responsible for the suppressive effects of the Ag and IL-2 stimulated T cells, it was still possible that the adoptively transferred activated T cells functioned by restricting Ag access or T cell stimulation. For instance, the activated suppressor cells may have induced pathways preventing naive T cells from engaging Ag present on APCs. To assess for this, we analyzed the responder T cells' up-regulation of the CD25 and CD69 activation markers in response to in vivo stimulation. When AND T cells were transferred into mice and left unstimulated, they did not proliferate and did not up-regulate either CD25 or CD69 at 24 or 72 h after transfer (Fig. 8, *A* and *B*). When the cells were stimulated with i.v. PCC, as in Fig. 4, they up-regulated CD25 and CD69 at 24 h, but this was not maintained and despite extensive proliferation, levels of CD25 and CD69 returned to baseline by 72 h. When 3A9 T cells were transferred and stimulated with HEL-peptide but not IL-2 before transfer of the CFSE-labeled AND T cells, an identical result was observed. As expected, when the 3A9 cells were stimulated with supplemental IL-2, they

blocked PCC-driven proliferation of the AND T cells. But interestingly, this impediment to cell cycle entry did not also block expression of CD25 or CD69. Both markers were up-regulated on the CFSE$^+$, PCC-specific T cells at 24 h, as they were on control cells stimulated in the absence of suppressive HEL-specific T cells. By day 3, levels of CD25 were diminished as with control samples. Levels of CD69, though diminishing in comparison with the 24 h time point, remained increased relative to controls. Therefore, naive T cells stimulated in the presence of IL-2/Ag activated T lymphocytes recognized cognate Ag on APCs. This recognition triggered an incomplete stimulation program, with up-regulation of CD25 and CD69 activation markers, but a failure to normally induce cell cycling.

T cells stimulated with Ag and IL-2 in vivo are not refractory or anergic, and do not produce suppressive cytokines

Cumulatively, our results show that T cells stimulated with Ag and supplemental IL-2 in vivo can suppress subsequent naive T cell responses. To better establish the mechanism of suppression of the Ag/IL-2 stimulated T cells, we analyzed their proliferation and cytokine production. AND T cells were adoptively transferred and left unstimulated or stimulated with PCC with or without IL-2. The splenocytes were isolated three days later, and the transferred AND T cells purified and stimulated with PCC again in culture. The AND T cells isolated from mice stimulated with Ag/IL-2 in vivo proliferated equivalently to freshly isolated naive AND T cells or T cells that had been transferred but left unstimulated or stimulated in the absence of IL-2 (Fig. 9*A*). They further produced equivalent levels of IL-2 (Fig. 9*B*). These results demonstrate that Ag or Ag/IL-2 stimulation of AND T cells in vivo does not induce unresponsiveness in them. Implicitly, these cells, which are suppressive in vivo, are not anergic, like suppressive Foxp3$^+$ Treg, or refractory like the suppressive cells generated after similar stimulation in vitro (Fig. 1 and Ref. 14).

To determine whether Ag/IL-2 stimulation altered the differentiation profile of the T cells, we also analyzed effector cytokine production. We were particularly interested in IL-10 and TGF-β secretion, because these cytokines have been shown to be important for the regulatory effects of Foxp3$^+$ Treg, Tr1, and Th3 T cells (27, 28). Little IL-10 was produced among any of the T cells, and there was no significant difference in production when comparing freshly isolated naive T cells, T cells transferred and left unstimulated, or T cells transferred and stimulated with Ag alone or Ag/IL-2 (Fig. 9*C*). TGF-β production was also undetectable among any of the AND T cell populations (data not shown). Therefore Ag/IL-2 treatment does not appear to convert the AND T cells into one of the previously defined populations of IL-10 or TGF-β-secreting Treg.

To determine whether other effector cytokines produced by the Ag/IL-2-treated T cells were altered, we also analyzed the production of a Th1 cytokine, IFN-γ, and a Th2 cytokine, IL-4 (Fig. 9, *D* and *E*). The AND T cells treated in vivo with Ag/IL-2 demonstrated increased production of both of these cytokines compared with control unstimulated or Ag-alone stimulated T cells, indicating that in contrast to IL-10 and TGF-β production, the supplemental IL-2 does promote secretion of effector cytokines by the AND T cells.

In summary, AND T cells stimulated in vivo with Ag in the presence of supplemental IL-2 do not secrete regulatory cytokines and are not anergic. Their effector cytokine profile is modestly altered by the IL-2 treatment, however, with increased secretion of both Th1 and Th2 cytokines.

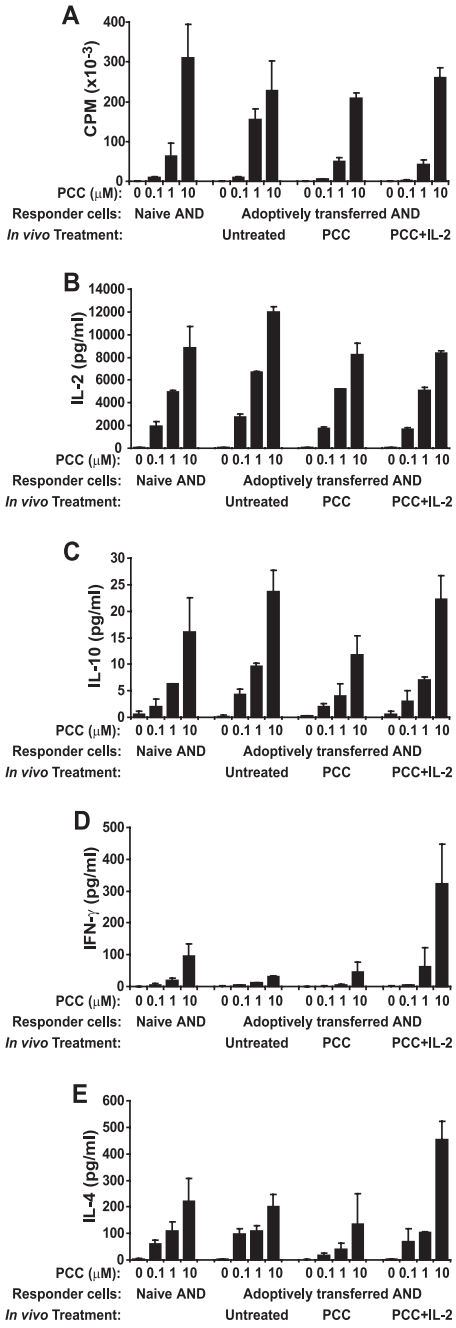

FIGURE 9. In vivo Ag/IL-2 stimulated T cells are not anergic and do not produce inhibitory cytokines. A total of 10^7 AND T cells were adoptively transferred and left unstimulated or stimulated with PCC with or without IL-2. Vβ3$^+$Vα11$^+$ AND T cells were purified by flow cytometric sorting from splenocytes 3 days later. These or control freshly sorted AND T cells were stimulated with the indicated concentration of PCC in the presence of irradiated APC. Proliferation (*A*) was measured at 72 h by [^{3}H]TdR incorporation. Cytokine production (*B–E*) was determined in the cell-free supernatant at 48 h by Bio-Plex.

Endogenous, but not AND Foxp3$^+$ Treg are preferentially expanded by Ag/IL-2

Considering the low IL-10 and undetectable TGF-β production by the Ag and IL-2-stimulated suppressive T cells, and their normal proliferative response in vitro, it was unlikely that expansion of Ag-specific Foxp3$^+$ lymphocytes was responsible for suppression. Nevertheless, IL-2 has been shown to expand Foxp3$^+$ Treg in vivo (29). Furthermore, Ag/IL-2 did induce

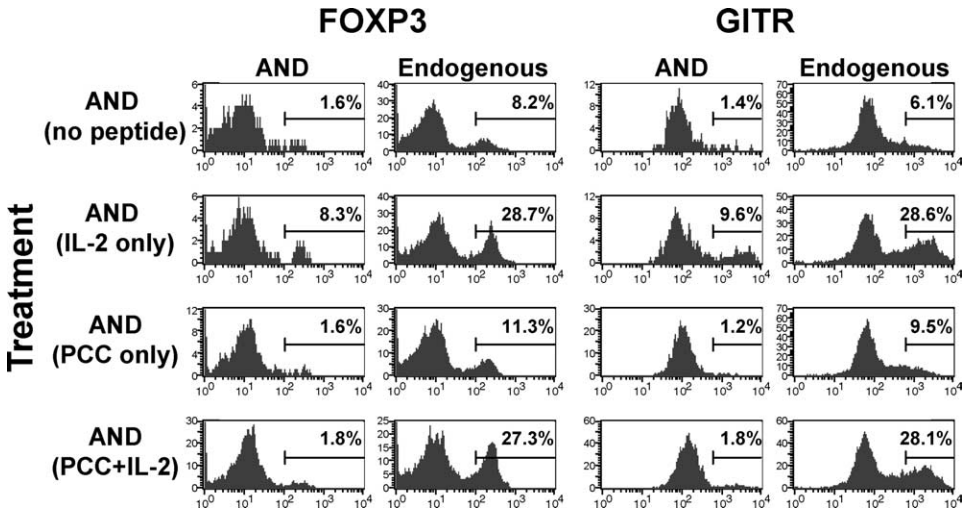

FIGURE 10. IL-2/PCC induces expansion of endogenous, but not AND, Treg. A total of 10^7 AND T cells were adoptively transferred and left unstimulated or stimulated with PCC and/or IL-2. $CD4^+V\beta3^+V\alpha11^+$ (AND) and $CD4^+V\beta3^-V\alpha11^-$ (endogenous) gated splenocytes were analyzed for Foxp3 or GITR 3 days later. Percent positive cells is indicated for either the AND T cell or endogenous T cell populations.

increased levels of CD25 (Fig. 4), a marker associated with both Treg and activated T cells. To more directly evaluate the induction of Treg by Ag/IL-2 treatment, we analyzed the expression of Foxp3 and GITR on adoptively transferred and stimulated AND T cells. We simultaneously evaluated endogenous host-derived T cells (Fig. 10). The percentage of $Foxp3^+$ T cells among transferred control unstimulated AND T cells was low, less than 2%, which is consistent with the high level of allelic exclusion of the AND TCR in AND Tg mice. This contrasted with an ~8% frequency of $Foxp3^+$ cells among endogenous $CD4^+$ T cells. Treatment of mice with IL-2 alone increased the percentage of $Foxp3^+$ transferred AND T cells ~4-fold. A similar fold increase in endogenous $Foxp3^+$ Treg was seen. Therefore, consistent with prior reports (29), IL-2 can expand $Foxp3^+$ Treg numbers. In contrast, stimulation of the AND T cells with PCC alone did not affect the percentage of $Foxp3^+$ T cells among either AND or endogenous T cells. Interestingly, unlike treatment with IL-2 alone, when the AND T cells were stimulated with PCC and IL-2, preferential expansion of the AND Treg population was not seen. The percentage of Treg was the same as in mice not receiving PCC or mice receiving PCC without IL-2. In contrast, the percentage of endogenous Treg did increase after treatment with PCC/IL-2 similarly to treatment with IL-2 alone. Therefore preferential expansion of Treg is observed to IL-2 alone, however, when T cells are Ag-stimulated both effector cells and Treg expand in similar proportions. Analysis of a second Treg marker, GITR, demonstrated results identical with that seen with Foxp3 staining (Fig. 10).

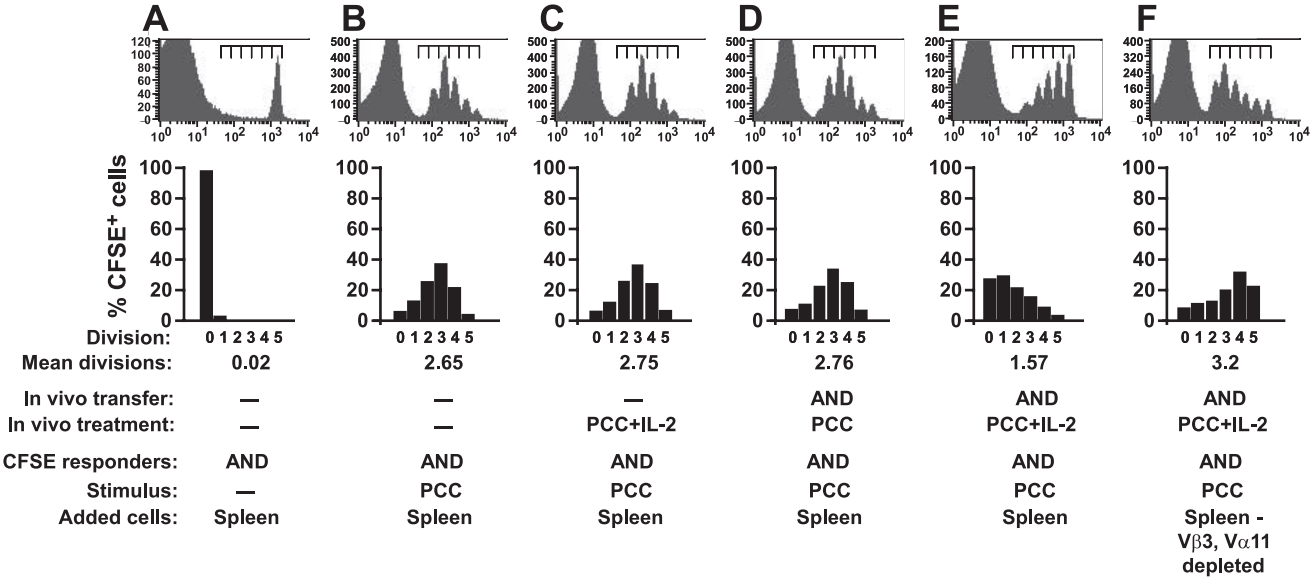

FIGURE 11. Suppression is not mediated by endogenous Ag/IL-2 stimulated T cells. A total of 10^7 AND T cells were adoptively transferred and left unstimulated or stimulated with Ag and/or IL-2. The spleens were removed from the mice 3 days later. A total of 3×10^5 non-irradiated splenocytes were added to 10^5 freshly-isolated CFSE-labeled $CD4^+CD25^-$ AND T cells, and these were cultured in the presence or absence of 5 μM PCC peptide. *A*, B10.BR splenocytes that had not received AND cells were added to CFSE-labeled AND T cells. The culture was not stimulated with PCC. Flow cytometric histogram showing CFSE dilution with proliferation is shown above corresponding plot depicting the percentage of CFSE labeled cells that underwent the indicated number of cell divisions. *B*, As in *A* but stimulation of culture with PCC. *C*, Splenocytes were from B10.BR mice treated with PCC/IL-2 only. *D*, Splenocytes from mice receiving AND cells and stimulated with PCC. *E*, Splenocytes from mice receiving AND cells and stimulated with PCC/IL-2. *F*, Splenocytes from mice receiving AND cells and treated with PCC/IL-2. The $V\beta3^+V\alpha11^+$ AND T cells were depleted from the splenocytes by flow cytometric sorting before their addition to the culture. Note that the modestly enhanced proliferation seen with the depleted splenocytes was not consistent across experiments.

These results demonstrate that PCC/IL-2 stimulation of AND T cells in vivo does not preferentially induce the expansion of Ag-specific Foxp3$^+$ Treg. It does, however, expand the endogenous Ag non-specific population of Treg. Importantly, our results do not support an essential role for these endogenous, Ag non-specific Treg in the suppression of Ag-specific AND responses. Ag/IL-2 treatment of mice in the absence of adequate doses of Ag-specific 3A9 or AND T cells has no effect on the proliferation of subsequently transferred and stimulated Ag-specific T cells (Fig. 6 and data not shown).

Suppression by Ag/IL-2 stimulated AND T cells is AND T cell dependent and is not mediated by endogenous Treg

IL-2 administration induced endogenous Treg expansion, though our data indicated that these cells were not responsible for suppression after adoptive transfer and Ag/IL-2 stimulation of AND or 3A9 T cells. To confirm this interpretation, we analyzed the ex vivo suppressive activity of splenocytes from mice receiving the various treatments in vivo. When purified naive CFSE-labeled AND T cells were admixed with non-irradiated splenocytes from unmanipulated B10.BR mice, they proliferated well in response to PCC (Fig. 11, *A* and *B*). If the splenocytes were obtained from mice treated with IL-2 and PCC, but not with AND T cells, an identical level of proliferation was seen (Fig. 11*C*). This shows that the increased proportion of endogenous Treg induced by IL-2 treatment is not associated with a diminished Ag-specific AND T cell response. Splenocytes isolated from mice that received AND T cells and PCC, but not IL-2, also did not influence the naive AND T cell response, paralleling our results after in vivo transfer of AND T cells (Fig. 11*D*). However, when splenocytes were isolated from mice in which AND T cells were transferred and stimulated with both PCC and IL-2, substantial inhibition of the proliferation of the CFSE-labeled cells was seen in vitro (Fig. 11*E*). Proliferation suppression was incomplete. However, it must be considered that the AND T cells constituted only ~7% of the 3×10^5 splenocytes added per 1×10^5 CFSE-labeled AND T cells in the culture, and therefore the ratio of "suppressor" to naive T cells was only ~1:5. To determine whether the in vivo activated AND T cells were required for this suppression, we depleted these cells from the splenocytes. Interestingly, the suppression was abrogated when the AND T cells were removed despite the continued presence of elevated levels of endogenous Foxp3$^+$ Treg (Fig. 11*F*). Therefore, adoptively transferred, Ag/IL-2 stimulated T cells are directly required to suppress subsequent naive T cell responses. In contrast, endogenous Treg are not directly suppressive.

Discussion

The mechanisms underlying repertoire selection during an immune response are incompletely understood. T cell-mediated suppression may conceivably play a role if it differentially inhibits T cells based on response characteristics, or alternatively based on temporal recruitment into the immune response. We provide proof-of-principle for such suppressive effects impacting immune responses. Specifically, adoptively transferred or in situ-generated IL-2- and Ag-activated CD4$^+$ T cells can inhibit subsequent naive T cell responses. Inhibition is not Ag- or MHC-restricted, demonstrating that the suppressed and suppressor T cells are not competing directly for Ag. Cross-competition, or competition among T cells specific for distinct epitopes, is well characterized in CD8 T cell responses (30). In contrast, competition across epitopes in CD4 T cell responses, which we observe here, has not previously been directly observed (1).

Our in vivo findings are largely in concordance with our prior in vitro observations with refractory T cell-mediated suppression (13, 14). IL-2- and Ag-stimulated T cells are able to suppress de novo T cell responses both in vitro and in vivo in an MHC/Ag-unrestricted manner. Furthermore, in each case the targeted T cells recognize Ag and up-regulate early activation markers despite their failure to enter cell cycle. One difference between our observations with in vitro and in vivo suppressive populations, however, is that whereas in vitro suppression correlates with T cell refractoriness (14), the suppressive T cells in vivo are not refractory (Fig. 9).

T cell-mediated suppression similar to that seen in this study has also been seen with other T cell types, most prominently anergic Foxp3$^+$ Treg (31), but also T cells that are anergic due to a lack of adequate costimulation (32, 33). T cell refractoriness resembles anergy, in that in each case T cells do not normally proliferate to TCR stimulation. Therefore, there is an association between suppressive activity and unresponsiveness among T cells. Considering this, unresponsiveness may be inherently linked to suppression. If this is the case, the difference in refractoriness between in vivo and in vitro Ag and IL-2-stimulated, suppressive T cells may suggest that they are biologically distinct and operate through different mechanisms. This interpretation would, however, seem unlikely. The induction requirements for the suppressive T cells in vitro and in vivo are highly similar, as are the manifestations of suppression. Further suggesting a common mechanism, suppressive activated T cells generated in vitro were able to suppress immune responses in vivo (Fig. 2), and suppressive cells generated in vivo were likewise active in vitro (Fig. 11).

A more likely explanation for the dissociation between refractoriness and suppression is that the refractoriness seen in vitro is not mechanistically linked to suppression, but is rather just an associated property. This would imply that antigenic stimulation in the presence of IL-2 independently induces both currently undefined suppressive mechanisms in T cells and stimulation refractoriness. Conceivably, due to differences in how IL-2 is administered (pulsatile dosing in vivo but not in vitro), the concentration of IL-2 the T cells encounter, or the differential availability of other cytokines or costimulatory molecules, refractoriness develops in vitro but does not develop in vivo despite the similar acquisition of suppressive functions by the activated T cells.

Whereas suppression mediated by anergic T cells is well documented, precedent for suppression mediated by activated T cells is not. An interesting example of this type of suppression is, however, potentially present in NF-κB-inducing kinase-deficient mice, in which T cells with an activated phenotype suppress the proliferation of otherwise hyperresponsive naive T cells (34). How the activated cells do this, however, remains unresolved.

In future studies it will be important to better delineate the suppressive mechanisms of activated T cells. The requirement for IL-2 to induce suppressive activity may provide a useful clue. Foxp3$^+$ Treg, like the activated T cells in this study, are also highly dependent on IL-2 (31). These Treg constitutively express high affinity IL-2R and are homeostatically dependent on IL-2 (35–37). IL-2 further stimulates suppressive activity within the Treg (38). A number of different suppressive mechanisms are used by Treg, including the production of adenosine, Lag3, granzymes, and IDO (39). These may therefore serve as candidates for directed assessments of the mechanism of suppression by Ag and IL-2 stimulated T cells. However, a complete picture of how Foxp3$^+$ Treg are able to suppress naive T cells has yet to emerge, and a more unbiased approach to a mechanistic analysis may be needed. Indeed, IL-2 has many roles in T cell activation, promoting proliferation, and both preventing apoptosis and enabling activation induced cell death (40–43), and the mechanisms by which IL-2 promotes suppression by activated T cells and Treg may be distinct.

Although we were not able to establish the mechanism(s) underlying suppression by the Ag and IL-2 stimulated T cells, our data do show that some commonly used regulatory mechanisms are not involved. We did not identify any significant increase in IL-10 or TGF-β production by the suppressive T cells, suggesting that these regulatory cytokines are not significant. Likewise, our data fail to identify a role for Ag-specific Foxp3$^+$ Treg in the suppression. In vitro generated suppressive refractory cells, which are derived from CD4$^+$CD25$^-$ T cells, such as those used in Fig. 1*A*, do not express detectable levels of Foxp3 (13). In vivo, we do not detect any change in the proportion of Foxp3$^+$, AND Tg T cells after they are stimulated with Ag and IL-2 compared with Ag alone (Fig. 10 and data not shown). These results strongly suggest that the suppression by the activated T cells in vivo, like in the in vitro situation, is not dependent on the preferential expansion of Ag-specific Foxp3$^+$ Treg. It must be noted that we are not able to exclude functional changes to Ag-specific Foxp3$^+$ Treg following the administration of IL-2 (38). However, considering the small number of these cells present after adoptive transfer, it would seem unlikely that they are responsible for the dramatic suppressive effect we observe.

In contrast to adoptively transferred Ag-specific Foxp3$^+$ Treg, the proportion of endogenous CD4$^+$Foxp3$^+$ T cells did increase substantially with IL-2 treatment in our analyses. However, suppression was not seen unless we also provided and activated an adequate dose of Ag-specific cells (Figs. 6 and 11). This indicates that the endogenous Foxp3$^+$ cells could not directly suppress Ag-specific responses. It remains possible that these endogenous Foxp3$^+$ cells played an indirect or supportive role in limiting the naive T cell responses. Dissecting any such indirect contribution in vivo would be difficult as the elimination of Foxp3$^+$ T cells provokes spontaneous T cell activation, autoimmunity, and inflammation that may confound analyses. Nevertheless, the simplest explanation of our results is that endogenous Foxp3$^+$ Treg do not play a significant role in activated T cell-mediated suppression, whereas activated Ag-specific T cells are essential.

IL-2 is not only important in normal immune responses but is currently clinically used as an adjunct therapy in the treatment of some tumors (44, 45). The administration regimen of IL-2 in our analyses, multiple doses over a several day period, parallels its common clinical administration. The consequences and mechanisms underlying IL-2 immunotherapy are not fully understood. Recent findings, which we corroborate here, demonstrating that therapeutic IL-2 administration increases numbers of Foxp3 Treg, has raised an appreciation for the potential of IL-2 as an immunomodulatory therapy (29, 46). Our results reveal an additional mechanism through which IL-2 may modulate immune responses. By potentiating the development of suppressive activated T cells, therapeutically administered IL-2 may repress nascent immune responses.

It is interesting that high IL-2 conditions both enhance CD25 expression on activated T cells and correlate with suppressive activity. The possibility that the activated CD25$^+$ T cells serve as a sink for IL-2, preventing exposure to IL-2 by and expansion of naive T cells would seem unlikely. Supplementation with IL-2, both in vitro and in vivo, when naive T cells are activated in the presence of suppressive cells does not rescue the expansion of the naive T cells. Furthermore, although IL-2 is an important promoter of cellular expansion both in vitro and in vivo, Ag stimulated T cells can proliferate in its absence (47, 48). In contrast, we observe oftentimes a complete proliferation block with T cell suppression, implying a distinct mechanism. It would therefore seem more probable that CD25 is a marker of, rather than a mechanism for, activated T cell-mediated suppression.

In summary, we demonstrate that activation of T cells in the presence of IL-2 suppresses the proliferation of, though not Ag recognition by, subsequently stimulated naive T lymphocytes. This suppression validates our prior in vitro observations regarding refractory T cell-mediated suppression in an in vivo system, provides the first identified mechanism for cross-competition among CD4$^+$ T lymphocytes, and suggests a novel negative feedback loop that may restrain overly vigorous immune responses. Such suppression may be important in the presence of strong immune responses and after the therapeutic administration of IL-2.

Acknowledgments

We gratefully acknowledge Richard Cross and Jennifer Smith for assistance with flow cytometric sorting and cytokine analysis.

Disclosures

The authors have no financial conflict of interest.

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