Adaptive Tolerance of CD4$^+$ T Cells In Vivo: Multiple Thresholds in Response to a Constant Level of Antigen Presentation

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The in vivo T cell response to persistent Ag contains a hyporesponsive phase following an initial expansion and subsequent partial deletion of the responding cells. The mechanism(s) responsible for this tolerance process is poorly understood. In this study, we describe a new paired transgenic model (TCR and Ag), which within 7–14 days produces 20–40 million hyporesponsive T cells. This state is characterized by an 85–95% reduction in all cytokine production, an impairment of re-expression of CD25 and CD69, and a desensitization of the proliferative response to Ag. TCR levels were normal, and in vivo mixing experiments showed no evidence for active suppression. The hyporesponsiveness partially dissipated without proliferation when the cells were transferred into a non-Ag-bearing host. If the second host expressed Ag, the T cells initially regained responsiveness, but then slowly entered an even deeper state of tolerance characterized by an additional 7- to 10-fold lowering of cytokine production and a greater desensitization of proliferation. Surprisingly, this readaptation took place with the same level of Ag presentation, suggesting that other parameters can influence the tolerance threshold. Both the readjustment in sensitivity and the reversal without Ag convincingly demonstrate for the first time a truly adaptive tolerance process in CD4$^+$ T cells in vivo. ***The Journal of Immunology,*** **2001, 167: 2030–2039.**

The response of naive T cells to Ag or superantigen in vivo entails an initial expansion phase, followed by deletion of a significant portion of the responding cells (1–4). Under many circumstances, the Ag quickly disappears and a small cohort of T cells remains that serves as a memory pool (5). Interestingly, under some conditions, the surviving T cells appear to be functionally hyporesponsive (anergic), as assessed by proliferation and cytokine production following restimulation in vitro (6–8). Over time these anergic T cells disappear or revert to a responsive state if the Ag is cleared (7, 9). However, if the Ag persists, the T cells also persist for some time and remain functionally unresponsive (7, 8, 10, 11). A similar scenario has been observed for B cell anergy in the absence of competing normal B cells (12).

The outcome of priming vs tolerance can be influenced by LPS administration or anti-CD40 Ab, stimuli that are known to activate APC and produce proinflammatory cytokines such as IL-6 and TNF-α (7, 13, 14). This suggests that the costimulatory environment present during initial Ag exposure might be critical for determining the eventual fate of the immune response. However, LPS can be given up to 2 days after introduction of the (super)Ag, demonstrating that the initial T cell encounter with an APC is not the sole fate-determining event. These observations suggest that naive T cells initially respond positively to the Ag, but then readjust their response potential several days later in an adaptive process that remains poorly understood (15). This re-evaluation step by the cell could be critical for the decision to die, become a memory cell, persist as an effector cell, or down-regulate responsiveness. Parameters such as the remaining Ag concentration, the persistence of costimulation (B7s, ICAM), the presence of CTLA-4 or programmed death-1, the levels and types of cytokines being produced (IL-10, TGF-β, IFN-γ, IL-4), the presence of regulatory T cells (Tr1, CD25$^+$), and the density of responding T cells could all be important variables in this decision-making process.

To explore some of these variables in a simplified model system, we studied the response of naive peripheral CD4$^+$ T cells to persistent Ag in vivo. A few such models already exist, but they use heterogeneous populations of T cells and/or have not been extensively examined during the adaptive phase (16–18). To create a better model, we transferred recombination-activating gene (Rag)[4]2$^{-/-}$ TCR transgenic (Tg) CD4$^+$ T cells specific for pigeon cytochrome *c* (PCC)/I-E^k (19, 20) into a second Tg mouse expressing the Ag under the control of an MHC class I promoter and an Ig enhancer (21). This second Tg had been previously crossed by us to a CD3$\epsilon^{-/-}$ mouse (22) to create a host with no T cells. This allowed us to follow the fate of the transferred, monospecific, naive CD4$^+$ T cell population in the absence of competition by other T cells. Interestingly, this model yielded an enormous population of hyporesponsive T cells whose intrinsic adaptive tolerance could be induced to different degrees, even though Ag presentation was kept constant at a very low level. These observations, plus the reversibility of the state, suggest that CD4$^+$ T cells can tune their threshold of responsiveness in the face of persistent Ag.

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[4] Abbreviations used in this paper: Rag, recombination-activating gene; LN, lymph node; mPCC, membrane-associated PCC; PCC, pigeon cytochrome *c*; Tg, transgenic.

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Materials and Methods

Mice

B10.A/SgSnAi (B10.A) mice and various knockout and Tg lines crossed onto that background were bred at the National Institute of Allergy and Infectious Diseases contract facility at Taconic Farms (Germantown, NY), an American Association for the Accreditation of Laboratory Animal Care-accredited specific pathogen-free barrier facility. The derivation of the B10.A TCR-Cyt 5C.C7 Rag2$^{-/-}$ mouse was described previously (20). This strain carries the Vα11/Vβ3 CD4$^+$ T cell Tg receptor specific for the PCC peptide 81–104 bound to I-E^k. This mouse will be referred to as the TCR-Tg. A second Tg mouse (called either RO or membrane-associated PCC (mPCC)) was the generous gift of S. Hedrick and S. Oehen (University of California, San Diego, CA) (21). This mouse expresses a membrane-targeted form of PCC under the control of an MHC class I promoter and an Ig enhancer. We received the mice on a B6 background at N5 and crossed them twice to B10.A, selecting for the MHC^a haplotype. We then crossed them with a CD3$\epsilon^{-/-}$ mouse (22), which we had also placed on a B10.A background with two backcrosses and an intercross. The final B10.A mPCC Tg CD3$\epsilon^{-/-}$ mouse generated will be called mPCC-CD3$\epsilon^{-/-}$. All mice used from this strain were heterozygous for the mPCC transgene. As a no Ag control in our experiments, the CD3$\epsilon^{-/-}$ mouse on a B10.A background was used (abbreviated as CD3$\epsilon^{-/-}$).

Transfer and retransfer of Tg cells

Naive T cells (3×10^6) isolated from the lymph nodes (LNs) (cervical, axillary, brachial, inguinal, and mesenteric) of TCR-Tg mice ($\geq$95% CD4$^+$ cells) were injected i.v. into mPCC-CD3$\epsilon^{-/-}$ or CD3$\epsilon^{-/-}$ mice. Before injection, the cells were labeled with 10 μM CFSE (Molecular Probes, Eugene, OR), as previously described (23). In one set of experiments, we also injected naive CFSE-labeled TCR-Tg cells i.v. into mPCC-CD3$\epsilon^{-/-}$ mice that had previously received unlabeled naive TCR-Tg cells 38 days before (full (D38) hosts).

For the retransfer experiments, mPCC-CD3$\epsilon^{-/-}$ mice were sacrificed 38 days after the injection of naive TCR-Tg cells. CD4$^+$ T cells were purified from LNs (>90% purity) by magnetic bead depletion of B cells, as well as macrophages and dendritic cells, using a two-step procedure. The first depletion was with sheep anti-mouse Ig-coated Dynabeads M-450 (Dynal, Lake Success, NY), and the second was with a mixture of rat mAbs (anti-Mac1, anti-B220, and anti-I-E^k; BD PharMingen, Mountain View, CA), followed by sheep anti-rat IgG-coated Dynabeads M-450. The TCR-Tg cells were then stained with CFSE and 3×10^6 injected i.v. into CD3$\epsilon^{-/-}$ mice, into mPCC-CD3$\epsilon^{-/-}$ mice, or back into full (D38) hosts. Spleen and LNs were removed from these mice and studied at different time points after the retransfer. In one experiment, the second hosts were injected i.p. at day 35 with 10 μg staphylococcal enterotoxin A (Toxin Technologies, Sarasota, FL) in PBS, and the cellular expansion was monitored after 2, 4, and 8 days.

Flow cytometry

Cell suspensions were stained with PE-labeled Abs: anti-CD4 (Caltag, San Diego, CA), anti-CD69, anti-CD25, and biotinylated anti-Vβ3 Abs revealed by streptavidin Tricolor (BD PharMingen). Immunofluorescence analysis was performed on a FACScan cytometer (BD Biosciences, Mountain View, CA), and data files were analyzed using CellQuest software (BD Biosciences).

T cell proliferation assay and cytokine ELISA

Five thousand cloned A.E7 T cells or 10,000 purified TCR-Tg CD4$^+$ cells were cultured with graded concentrations of PCC peptide (aa 81–104, synthesized and HPLC purified by the National Institute of Allergy and Infectious Diseases core facility (National Institutes of Health, Rockville, MD)) and 5×10^5 irradiated (3000 rad) spleen cells as APC from CD3$\epsilon^{-/-}$ mice or mPCC-CD3$\epsilon^{-/-}$ mice. In some experiments, normal B10.A spleen cells were anti-Thy-1 depleted, irradiated, and used as APC. In one experiment, the TCR-Tg CD4$^+$ cells were preactivated by stimulating them with PCC and B10.A splenic APC for 48 h, expanding them in 10 U/ml IL-2, and using them after 5 days of rest. The culture medium was RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FCS (Biofluids), 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. The cultures were set up in flat-bottom, 96-well plates (Costar 3596, Corning, NY). After 48 h of culture, 100 μl of supernatant was removed for cytokine assays, and the concentrations of all the cytokines were determined by sandwich ELISA (Endogen, Woburn, MA, or R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The wells were then pulsed with 1 μCi [^{3}H]TdR (6.7 Ci/mmol; ICN Biomedical, Costa Mesa, CA) and harvested 16 h later. The incorporated [^{3}H]TdR was measured by scintillation counting in a Betaplate 1205 detector (Wallac, Gaithersburg, MD). Dose-response curve data were fit with the nonlinear regression software program of Prism GraphPad, and the EC_{50} were calculated. To determine the ability of the cells to re-express CD25 and CD69, they were cultured for 48 h under the same conditions, collected, and then stained for flow cytometry analysis, as described above.

Results

Naive CD4$^+$ T cells respond rapidly and completely to mPCC-CD3$\epsilon^{-/-}$ APC in vivo

CD4$^+$ TCR-Tg T cells were collected from the LNs of naive mice and labeled in vitro with CFSE, and 3×10^6 cells were transferred i.v. into mPCC-CD3$\epsilon^{-/-}$ mice. One day later, 10–15% of the cells could be recovered from spleen and peripheral LNs (Fig. 1, *left graph*). These cells were all activated, as assessed by size enlargement measured using flow cytometry (data not shown) and CD25 and CD69 expression (see below). The cells began to proliferate between days 1 and 2 in vivo, and 80% of them had divided two or three times (Fig. 1, *right graph*, mPCC-CD3$\epsilon^{-/-}$). A similar doubling time (7–12 h per cycle) was maintained between days 2 and 3. After day 3, the expansion phase abated, reaching a maximum of 97-fold on day 4 (Fig. 1, *left graph*). However, after day 4 the T cell recovery began to decline, suggesting either the onset of apoptosis, as has been described in other model systems (24), or migration to other tissues. The cell loss in this second phase of the response was ~50%, and was completed by day 10–14. This was followed by a small increase in cell number over the next 6 wk. In this third phase, which can start as early as day 7 to 10, the cells have greatly curtailed their in vivo responsiveness to the Ag. We will refer to this stage as the adaptive phase.

These results contrasted with the behavior of the T cells in the absence of Ag. When the CD4$^+$ cells were transferred into a CD3$\epsilon^{-/-}$ host not expressing the Ag, they failed to expand or divide during the first week (Fig. 1, *left panel* for cell number and *right panel* labeled CD3$\epsilon^{-/-}$ for CFSE staining), even though in this particular experiment there were slightly more cells recovered at day 1 than from the mPCC-CD3$\epsilon^{-/-}$ host. However, starting at day 7, one division was observed in a small subset (20%) of the cells. A detectable increase in cell yield was seen by day 14, and by day 22, 75% of the cells had divided at least once and one-third of them more than once. By 2 months, the net expansion was ~4-fold. This response is similar to the homeostatic T cell proliferation observed by others in T cell-deficient hosts, which varies in magnitude and time of onset depending on the particular Tg TCR being studied (25). In our experiments, this phenomenon appears to be completely supplanted by the expansion and deletion phases of the T cell response to cognate Ag.

We also looked at the expression of early activation markers on the CD4$^+$ T cells isolated at different times after transfer into the mPCC-CD3$\epsilon^{-/-}$ hosts (Fig. 2). At 24 h, all the cells expressed high levels of CD69, and 85% of them had up-regulated CD25. After this initial full activation, both CD69 and CD25 levels were rapidly down-regulated, and near the end of the expansion phase (day 3) and during the deletion phase (day 8), these markers were no longer detected at the cell surface. However, in the adaptive phase (from day 14 onward), 30–40% of the CD4$^+$ T cells re-expressed CD69 (only day 38 is shown in Fig. 2), although the level of expression never achieved that observed on day 1. None of the cells re-expressed CD25 (Fig. 2), but most were CD44^{high} (data not shown).

CD4$^+$ T cells are hyporesponsive in the adaptive phase

The adapted T cells, isolated 38 days after transfer, were restimulated in vitro for 2 days with different amounts of PCC peptide

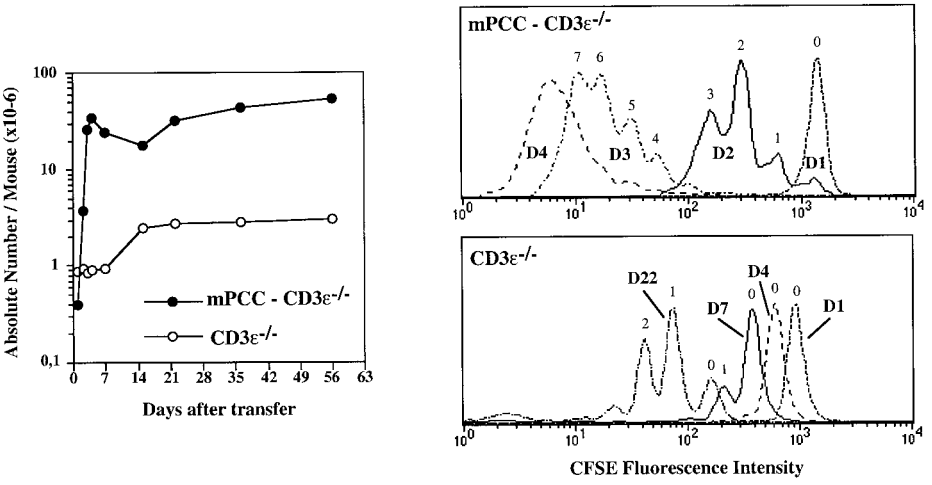

FIGURE 1. The role of Ag stimulation in naive TCR-Tg CD4$^+$ T cell expansion in vivo. A total of 3×10^6 CFSE-labeled naive TCR-Tg cells was injected into either mPCC-CD3$\epsilon^{-/-}$ mice (●) or CD3$\epsilon^{-/-}$ mice (○). The spleen and LNs were removed at various times after transfer and stained with anti-Vβ3 and anti-CD4. The total number of TCR-Tg CD4$^+$ cells recovered per mouse is shown at different time points after transfer, starting at day 1. The values were determined by multiplying the percentage of Vβ3$^+$, CD4$^+$ cells by the total number of cells in the spleen and LNs (*left graph*). The frequency histograms on the *right* represent the number of cell divisions of the TCR-Tg cells evaluated by the intensity of CFSE labeling at different time points. For the *upper histogram* (mPCC-CD3$\epsilon^{-/-}$), the times were day 1, 2, 3, and 4; for the *lower histogram* (CD3$\epsilon^{-/-}$), the times were day 1, 4, 7, and 22. The small numbers (0–7) on all the histograms represent the number of divisions that had taken place at each time point, as estimated by the loss of CFSE-staining intensity. Note that the 0 point shifts to lower intensity with time, because of a slow loss of the dye from the cells independent of division. The results shown in the *left graph* are averages from a pool of two mice per time point and are representative of one of two or three experiments performed.

and examined in this individual cell assay for their ability to up-regulate CD69 and CD25 (Fig. 3). Stimulation with 3 nM exogenous peptide did not induce detectable increases of CD69 or CD25 on the adapted T cells, whereas 50–60% of the naive T cells responded to the same concentration. In fact, the low levels of CD69 on the freshly isolated adapted T cells disappeared in culture. The adapted T cells did show a substantial CD69 and CD25 response (40%) at 100 nM, but even at very high concentrations of Ag (1 μM) only 70% of them re-expressed CD25, and only 50% significantly up-regulated CD69. The response patterns seen at these high doses for the adapted cells resemble those seen for the naive T cells at the low Ag doses (3–10 nM), suggesting a 100- to 300-fold shift in the Ag dose-response curves.

Proliferative responses to Ag were also measured in vitro after 48 h of stimulation (Fig. 4). One day after transfer, the maximum proliferative response was 6-fold greater than that observed with naive T cells, although the EC_{50} values were similar (9 nM for naive vs 7 nM for day 1) (Fig. 4*A*). This result suggests that activation in vivo is a very effective means of preparing many CD4$^+$ T cells to subsequently enter cell cycle in vitro without changing the Ag threshold for activation. However, after 7 days in vivo the T cells became hyporesponsive. The EC_{50} was shifted to 50 nM, and the maximum response returned to the level of naive cells. This 5-fold shift is maintained out at 36 days (EC_{50} = 42 nM) (Fig. 4*B*). In contrast, if the T cells were transferred into a CD3$\epsilon^{-/-}$ host not expressing Ag, the cells became hyperresponsive (EC_{50} = 1 nM) by day 36. This suggests that the homeostatic response leads to a memory-like state, whereas the response to persistent Ag leads by 7 days to an anergic-like state.

The cytokine responses support this conclusion (Fig. 5). On day 7, IL-2 production by adapted T cells in response to 1 μM peptide was 15% of that observed for naive T cells (Fig. 5*A*), and this hyporesponsiveness was maintained out to 36 days (Fig. 5, *A* and *C*). Naive T cells did not produce very much IL-4, IL-10, and IFN-γ following stimulation in vitro for 48 h with 1 μM peptide. However, after 24 h in the mPCC-CD3$\epsilon^{-/-}$ host, an enormous induction event occurred in the T cells, allowing them to produce

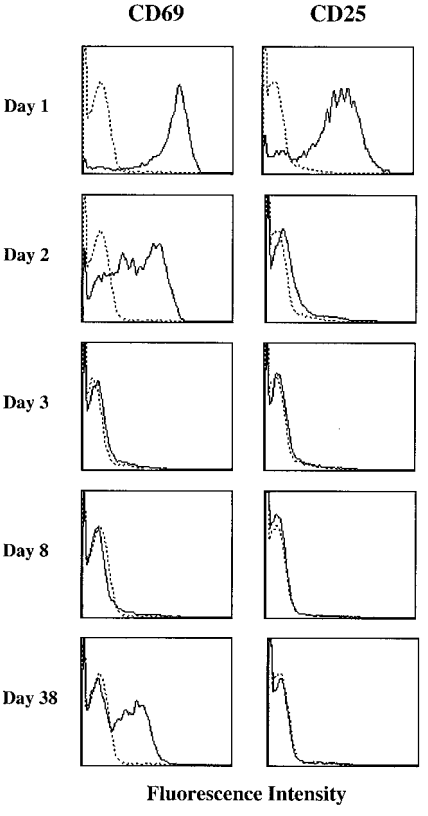

FIGURE 2. The appearance of CD69 and CD25 molecules on the surface of CD4$^+$ TCR-Tg cells activated in vivo. Spleen and LN cells from mPCC-CD3$\epsilon^{-/-}$ mice injected with naive TCR-Tg cells were removed at various times after transfer (days 1–38); stained with anti-Vβ3, anti-CD69 or anti-CD25; and analyzed by flow cytometry. The frequency histograms show the CD69 or CD25 expression by naive TCR-Tg cells (dotted lines) or TCR-Tg cells recovered from mPCC-CD3$\epsilon^{-/-}$ host mice at various times after transfer (solid lines).

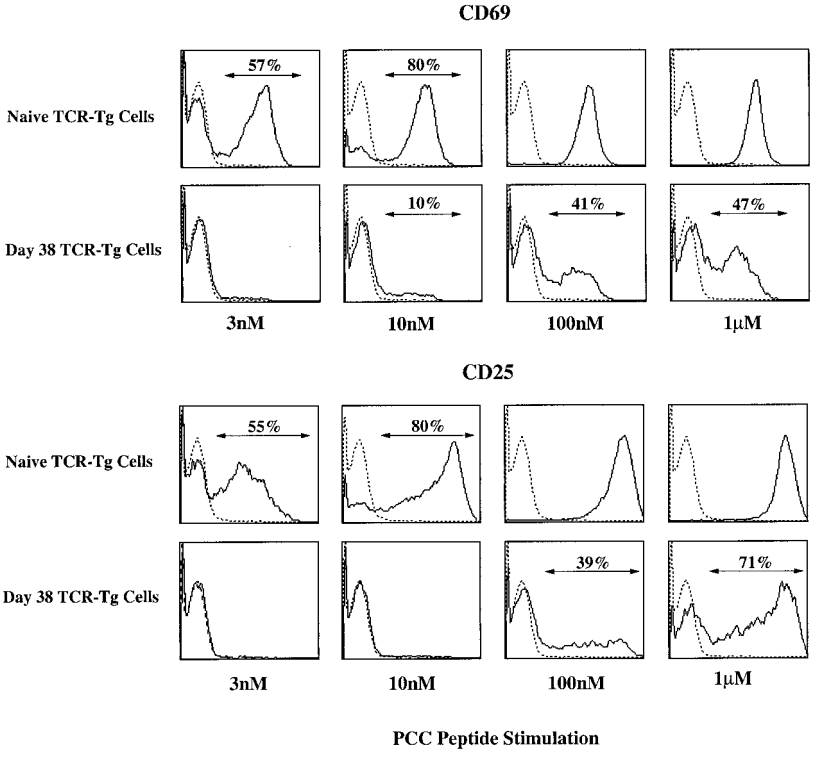

FIGURE 3. $CD4^+$ TCR-Tg cells in the adaptive phase are desensitized for CD69 and CD25 re-expression. Naive TCR-Tg cells or TCR-Tg cells recovered 38 days after transfer into mPCC-$CD3\epsilon^{-/-}$ mice were stimulated in vitro with Ag and APC for 48 h, as described in *Materials and Methods*, and then stained with anti-Vβ3, anti-CD69, or anti-CD25. The frequency histograms show the CD69 or CD25 expression by TCR-Tg cells stimulated without PCC peptide (dotted lines) or with increasing concentrations of PCC peptide (solid lines).

large amounts of these cytokines. IFN-γ production was the greatest (Fig. 5*A*), rivaling that produced by T cell clones such as A.E7. IL-4 and IL-10 production were also substantial (Fig. 5*B*). However, 7 days after transfer, all the cytokine production potentials were greatly reduced, and this reduction was maintained out to day 36. By this time, IFN-γ production was only 5% of that at day 1. IL-4 production was reduced to 5%, IL-3 production to 3%, and IL-10 production to 2% (Fig. 5, *A* and *B*). IL-3 production was also decreased relative to that of naive T cells (9.5%). Because the IFN-γ response was initially so large, the population remaining at day 36 gives the impression that the T cells have differentiated predominantly toward a Th1 phenotype. However, with regard to the adaptation process, it is clear that the degree of down-regulation is comparable for all the cytokines.

When the T cells were placed in a $CD3\epsilon^{-/-}$ host not expressing the Ag, no difference from naive cells was noted out to day 7 (data not shown). However, by 14 days the ability to produce significant amounts of IFN-γ was detected, and by 36 days the levels were comparable with those seen in the T cells adapted to the PCC Ag (Fig. 5*D*). At this time point, though, a dose-response curve shows that in fact the two populations are quite different. Although the adapted cells required >10 nM peptide to elicit a detectable IFN-γ response, the homeostatically expanded population was still giving a strong response at concentrations as low as 3 nM. For IL-2 production (Fig. 5*C*), these cells were also slightly hyperresponsive in comparison with naive cells (EC_{50} = 130 nM vs 330 nM). Other cytokine production was either undetectable (IL-10) or only slightly more than that of the naive cells (IL-3 and IL-4) (data not shown). Thus, again, it appears that the homeostatic response leads to a hyperresponsive state, whereas the persistent PCC Ag stimulation leads to a hyporesponsive state.

Adaptation in vivo is not a change in Ag presentation

The initial immune response during the first week after transfer could potentially have led to destruction of the APCs in the mPCC-$CD3\epsilon^{-/-}$ host. If this process continued into the adaptive phase, then it might have resulted in a new steady state level of processed Ag, which was inadequate to strongly stimulate the T cells. Two approaches were used to examine this possibility. First, spleen cells were taken from either a naive mPCC-$CD3\epsilon^{-/-}$ mouse or a host containing day 40 or day 56 adapted T cells and used to stimulate either the T cell clone A.E7 or preactivated $CD4^+$

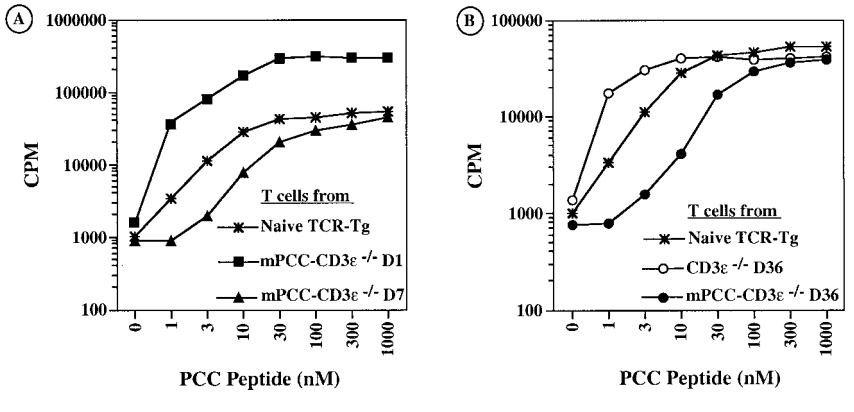

FIGURE 4. Proliferative responses to Ag in vitro by the adapted TCR-Tg cells. *A*, The in vitro proliferative responses at 48–64 h to various concentrations of PCC peptide by TCR-Tg cells isolated 1 (■) or 7 (▲) days after transfer into mPCC-$CD3\epsilon^{-/-}$ mice are compared with the proliferative response of naive TCR-Tg cells (∗). *B*, The proliferative response of TCR-Tg cells isolated 36 days after transfer into either mPCC-$CD3\epsilon^{-/-}$ mice (●) or $CD3\epsilon^{-/-}$ mice (○) is compared with the proliferative response of naive cells (∗). Results in *A* and *B* are the mean cpm of triplicate cultures.

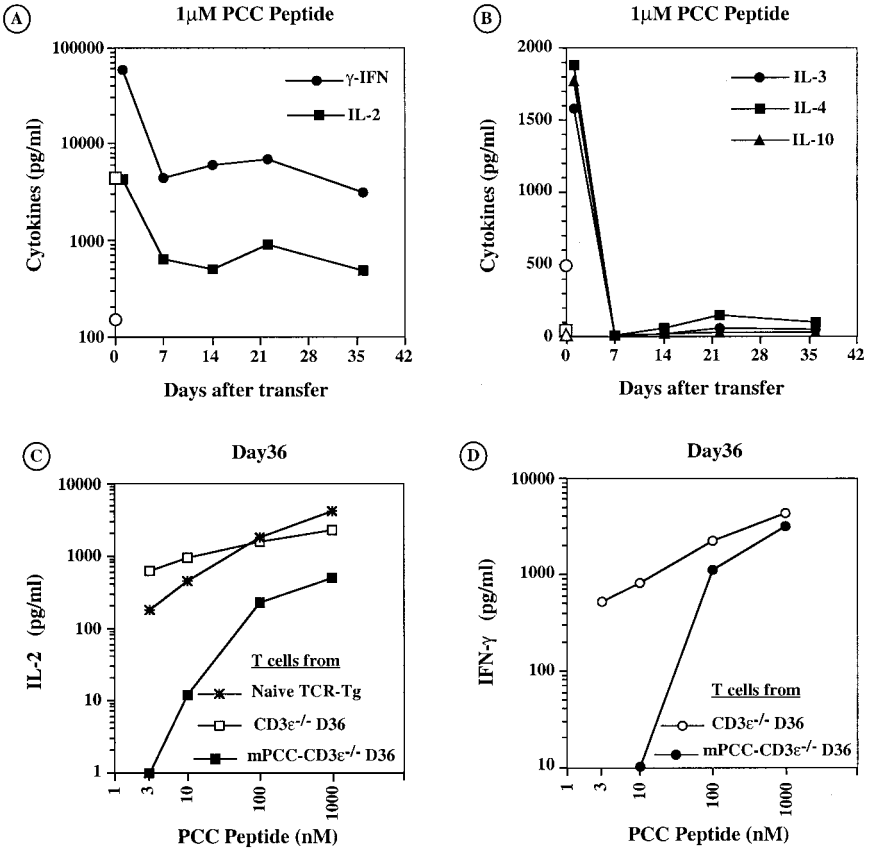

FIGURE 5. Cytokine production in response to Ag in vitro by the adapted TCR-Tg cells. *A* and *B*, Cytokine production by TCR-Tg cells isolated at various time points after transfer into mPCC-CD3$\epsilon^{-/-}$ mice (closed symbols) is compared with naive cells (open symbols), after 48 h of in vitro stimulation with 1 μM PCC peptide. *A*, Shows the kinetic curves for IL-2 (■, □) and IFN-γ (●, ○). *B*, Shows the kinetic curves for IL-3 (●, ○), IL-4 (■, □), and IL-10 (▲, △). *C* and *D*, Cytokine dose-response curves for IL-2 (■, □) and IFN-γ (●, ○) production by TCR-Tg cells isolated 36 days after transfer into mPCC-CD3$\epsilon^{-/-}$ mice (closed symbols) or CD3$\epsilon^{-/-}$ mice (open symbols), after in vitro stimulation with increasing concentrations of exogenous PCC peptide. The IL-2 response of naive TCR-Tg cells is shown for comparison (*) in *C*. Cytokine concentrations present in the supernatants at 48 h were evaluated by ELISA. The data are representative of two or three independent experiments.

TCR-Tg T cells. As shown in Fig. 6*A*, the magnitude of the T cell proliferative response in the absence of any added Ag was comparable for the two types of APC and similar to that obtained when 0.3 nM peptide was added to normal B10.A spleen cells. Furthermore, addition of exogenous PCC augmented the response of both mPCC APCs with similar dose-response curves, suggesting that the endogenous levels of processed Ag are the same in both mice. The transition point for augmentation of the endogenous response was carefully measured in Fig. 6*B* and found to occur for both APC between 0.08 and 0.16 nM, emphasizing the relatively low amount of PCC that is functionally expressed in vivo.

In the second approach, a cohort of naive CD4+ T cells was labeled with CFSE and injected into an mPCC-CD3$\epsilon^{-/-}$ mouse that had been reconstituted 38 days previously with Tg T cells and now contained 20–40 $\times$ 10^6 adapted CD4+ cells. As shown in Fig. 7, the CFSE-labeled second cohort expanded at the same rate and to the same magnitude as if they had been injected into an empty mPCC-CD3$\epsilon^{-/-}$ host (see Fig. 1 for comparison). This

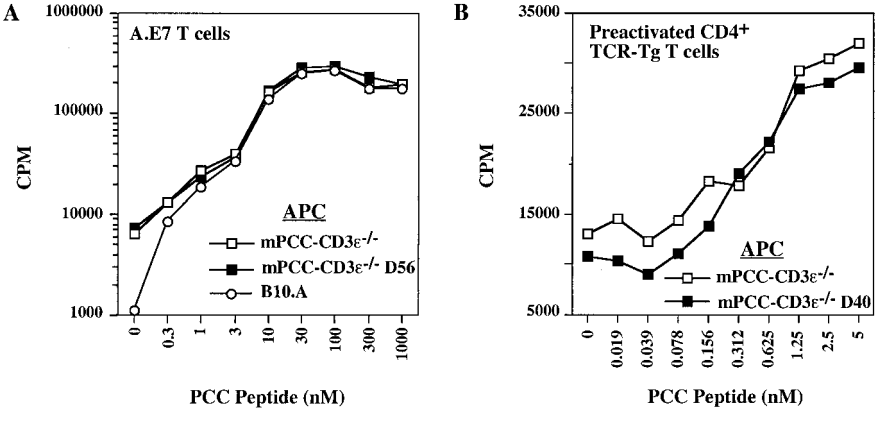

FIGURE 6. Ag presentation is the same for APCs from naive or adapted mPCC-CD3$\epsilon^{-/-}$ mice. *A*, Cloned A.E7 T cells were cultured in vitro, with or without 3-fold increasing concentrations of exogenous PCC peptide, in the presence of B10.A (○) or mPCC-CD3$\epsilon^{-/-}$ (□) splenic APCs from noninjected mice or splenic APCs from mPCC-CD3$\epsilon^{-/-}$ mice injected 56 days previously with TCR-Tg cells (■). *B*, Previously activated CD4+ TCR-Tg T cells were cultured in vitro with or without 2-fold increasing concentrations of exogenous PCC peptide, in the presence of either mPCC-CD3$\epsilon^{-/-}$ splenic APCs from noninjected mice (□) or splenic APCs from mPCC-CD3$\epsilon^{-/-}$ mice injected 40 days previously with TCR-Tg cells (■). Both *graphs A* and *B* show the proliferative responses of the T cells measured from 48 to 64 h, as evaluated by [^{3}H]TdR incorporation, and each point represents the mean cpm of triplicate cultures.

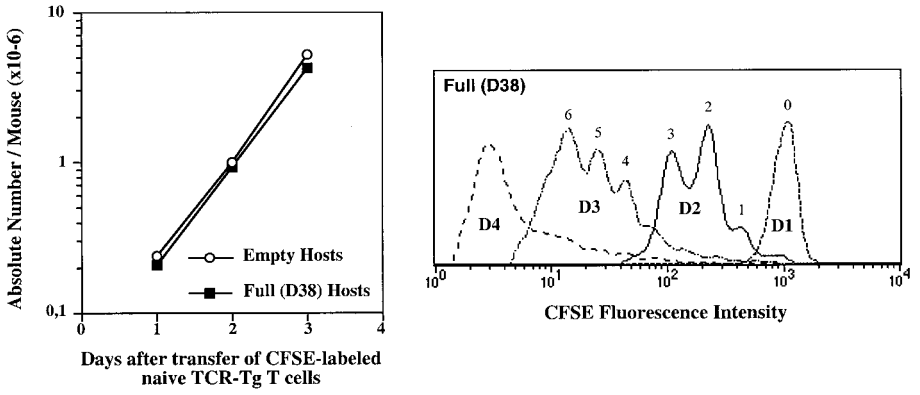

FIGURE 7. Naive CSFE-labeled TCR-Tg T cells injected into adapted mPCC-CD3$\epsilon^{-/-}$ hosts reveal normal levels of Ag presentation in vivo and no evidence for active suppression or Ag-specific receptor competition during proliferative expansion. Three million CFSE-labeled naive TCR-Tg cells were injected into either mPCC-CD3$\epsilon^{-/-}$ mice (○, empty hosts) or mPCC-CD3$\epsilon^{-/-}$ mice injected 38 days previously with an initial set of unlabeled naive TCR-Tg cells (■, full (D38) hosts). The *left graph* shows the total number of $CD4^+$ TCR-Tg T cells recovered per mouse at different time points after transfer. The frequency histogram on the *right* represents the intensity of CFSE labeling of the T cells recovered from the full (D38) hosts at day 1, 2, 3, or 4 after transfer. See Fig. 1 for a more detailed explanation.

experiment shows that the Ag presentation in vivo has remained the same. Furthermore, there appeared to be no competition or suppression from the adapted T cells in the tolerant host. This rules out a role for active immunoregulation as a mechanism to explain the proliferative unresponsiveness.

The possibility that the hyporesponsiveness of the adapted T cells was caused by a down-regulation of their TCRs was examined by staining the cells with anti-Vα11, anti-Vβ3, or anti-CD4. Little or no reduction of each of these molecules was detected (0–25%). Furthermore, occasional slight reductions were also seen on T cells that had come out of the state (see below), and thus there was no correlation with the functionality of the cells (data not shown).

Reversal of adaptation on retransfer of the T cells to a host not expressing the Ag

To determine whether the tolerant state was reversible, and thus truly adaptable, $CD4^+$ T cells were purified from LNs of day 38 adapted mPCC-CD3$\epsilon^{-/-}$ mice, and 3×10^6 of them (>90% purity) were injected into a CD3$\epsilon^{-/-}$ mouse not expressing the Ag. For the first 7 days, these cells did not divide at all (Fig. 8*A* and CFSE data not shown). Between days 7 and 36, a slow expansion occurred, culminating in a 6- to 8-fold increase in cell number, which was maintained out to day 56 (Fig. 8*B*). This pattern appeared similar to that of naive T cells in a CD3$\epsilon^{-/-}$ host (Fig. 1).

The in vitro proliferative responsiveness of the retransferred T cells was assessed from the time of transfer out to 56 days (Fig. 9*A*). Within 1 day after transfer, the proliferative dose-response curve had returned to the normal pattern seen for naive T cells. Interestingly, by 7 days, the cells had become hyperresponsive (EC_{50} = 2 nM vs 10 nM for naive cells). This increased sensitivity remained out to 56 days (EC_{50} = 3 nM). The ability to produce cytokines in vitro was also assessed after retransfer (Fig. 9*B*). Both IL-2 and IFN-γ production increased with time. For IFN-γ, the maximum response increased 5.7-fold by day 56 in experiment 1 (Fig. 9*B*) or 3.6-fold by day 38 in experiment 2 (Fig. 9*C*). For IL-2, the maximum response increased significantly by day 7, but did not reach the level of production of naive T cells until day 56 (Fig. 9*B*). The IL-2 EC_{50} returned to that of the naive cells by day 38 (Fig. 9*C*). For IFN-γ, the EC_{50} decreased only slightly (2-fold) by day 38, compared with the initial value at the time of retransfer (Fig. 9*C*). These results suggest that the hyporesponsive state can reverse when the cells are transferred into an environment that lacks the Ag. This reversal is partially accomplished in the first week, suggesting that the cell divisions that occur beyond day 7 (Fig. 8*B*) are not essential to initiate this change in responsiveness.

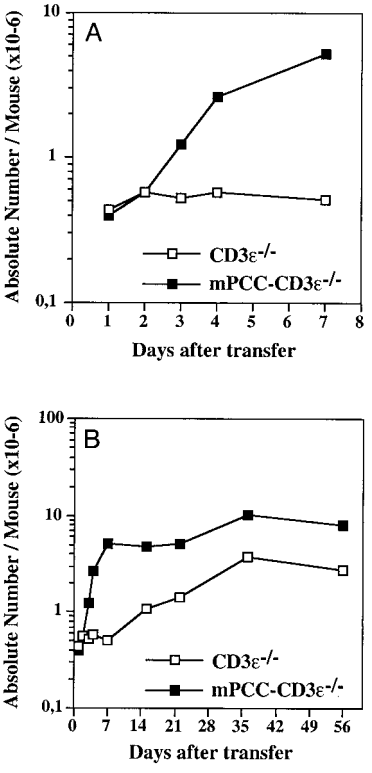

FIGURE 8. Cell expansion of the adapted TCR-Tg cells after retransfer into hosts expressing or not expressing the Ag. Three million purified, TCR-Tg cells isolated 38 days after transfer into mPCC-CD3$\epsilon^{-/-}$ mice were CFSE labeled and reinjected into either mPCC-CD3$\epsilon^{-/-}$ mice (■) or CD3$\epsilon^{-/-}$ mice (□). Results show the total number of TCR-Tg T cells recovered per mouse at different time points after transfer, calculated as described in Fig. 1. *A*, Results during the first week after transfer. *B*, Total results from days 1–56 after transfer. Data are means from a pool of two mice per time point for one of two (or for some time points, three) experiments.

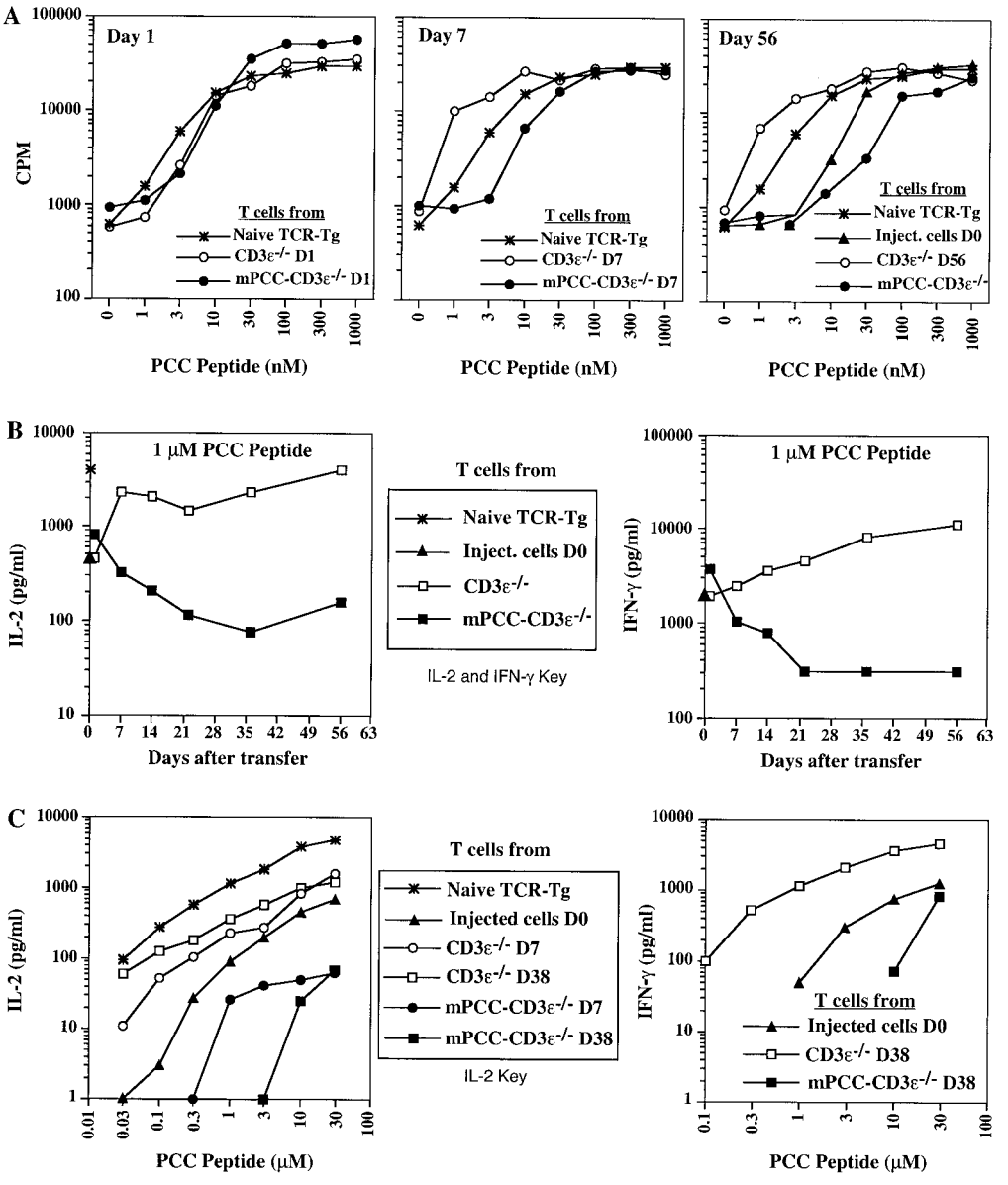

FIGURE 9. The adaptive state is reversible in the absence of Ag, but enhanced if the TCR-Tg cells are re-exposed to the same concentration of Ag in vivo. The proliferative and cytokine responses to PCC peptide of TCR-Tg cells retransferred after 38 days residing in an mPCC-CD3$\epsilon^{-/-}$ host into either a second mPCC-CD3$\epsilon^{-/-}$ host expressing the same amount of Ag or into a CD3$\epsilon^{-/-}$ host not expressing the Ag. See Figs. 4 and 5 for methodological details. *A*, The graphs show the proliferative responses, following 48- to 64-h in vitro stimulation with increasing concentrations of PCC peptide, by TCR-Tg cells isolated at day 1, 7, or 56 after retransfer into either mPCC-CD3$\epsilon^{-/-}$ mice (●) or CD3$\epsilon^{-/-}$ mice (○) as compared with naive TCR-Tg cells (*) or the day 38 TCR-Tg cells before retransfer (▲, inject. cells D0). Data points are the mean cpm of triplicate cultures. *B*, The graphs show the IL-2 (*left*) or IFN-γ (*right*) production, after in vitro stimulation with 1 μM PCC peptide, by TCR-Tg cells isolated at different time points after retransfer into either mPCC-CD3$\epsilon^{-/-}$ mice (■) or CD3$\epsilon^{-/-}$ mice (□) as compared with naive TCR-Tg cells (*) or day 38 TCR-Tg cells before retransfer (▲, inject. cells D0). The results in *A* and *B* are from the same experiment. *C*, In a second experiment, in which the in vitro culture conditions were less optimal than the first, dose-response curves were generated for IL-2 (*left graph*) and IFN-γ (*right graph*) production, using concentrations of PCC peptide ranging from 0.03 to 30 μM. The results shown are for TCR-Tg cells isolated at day 7 (circles) or day 38 (squares) after retransfer into either mPCC-CD3$\epsilon^{-/-}$ mice (●, ■) or CD3$\epsilon^{-/-}$ mice (○, □) as compared with naive TCR-Tg cells (*) or day 38 TCR-Tg cells before retransfer (▲, injected cells D0).

Enhancement of the tolerant state on retransfer of the T cells into an empty host expressing the Ag

The in vivo response to Ag of day 38 adapted T cells was also examined by injecting them back into day 38 adapted mice or into naive mPCC-CD3$\epsilon^{-/-}$ mice, both expressing the Ag. In the former host, with resident tolerant cells, no proliferative expansion of the new cohort was seen out to 7 days (data not shown). Surprisingly, in the naive mPCC-CD3$\epsilon^{-/-}$ empty host, the cells began to expand after day 1, and the proliferation lasted for several days (Fig. 8*A*). By 7 days, the new population had expanded 13-fold. This level was maintained out to day 56 (Fig. 8*B*). In comparison with naive TCR-Tg CD4+ cells (Fig. 1), the T cells adapted for 38 days were clearly hyporesponsive: the rate at which they doubled was only once every 40 h vs 7–12 h for naive cells. Furthermore, the maximum expansion was ~4-fold less. Thus, the T cells stopped dividing before the population had completed filling up the host. Finally, the fact that this expansion did not occur on injection back into a day 38 host, which already contained adapted

T cells, suggests that it is influenced by some parameter(s), such as cell density, in addition to the presence of the Ag.

A functional analysis of the cells retransferred into the empty mPCC-CD3$\epsilon^{-/-}$ host is shown in Fig. 9. The in vitro proliferative dose-response curve initially reverted to that of a naive population (Fig. 9*A*, day 1), but by day 7 the cells had again become hyporesponsive (EC_{50} = 24 nM). Interestingly, this decreased sensitivity appeared to become even greater after the cells stopped proliferating (EC_{50} = 83 nM at day 56), and in fact surpassed the hyporesponsiveness originally displayed by the transferred cells at the time of retransfer (EC_{50} = 30 nM). The acquisition of a deeper tolerant state was also seen for cytokine production. The levels of both IL-2 and IFN-γ produced in response to 1 μM peptide in vitro initially increased 1 day after transfer into the second mPCC-CD3$\epsilon^{-/-}$ host, but then declined slowly thereafter (Fig. 9*B*), reaching a 7- to 10-fold lower level of production by days 22–36 as compared with at the time of retransfer (injected cells D0). In the second experiment, shown in Fig. 9*C*, one can also observe from the dose-response curves that the remaining responding cells became more hyporesponsive with time. For IL-2 production, the curve shifted to higher Ag concentrations by 5- to 10-fold between days 7 and 38. For IFN-γ, a similar shift was seen between the initial dose-response curve at the time of retransfer and that observed at day 38. Production of IL-4 and IL-3 also diminished after retransfer (data not shown). These experiments demonstrate that the level of adaptive tolerance can be greatly influenced by environmental parameters other than just the expressed levels of Ag and costimulation.

Discussion

Although several investigators have examined the response of TCR-Tg T cells transferred into a second Tg, Ag-bearing host to study peripheral tolerance induction (4, 10, 15, 18), the new system we have developed has a number of unusual features and strengths that make it especially suited for studying the hyporesponsive state of a truly monospecific (Rag2$^{-/-}$) CD4$^+$ population of cells following Ag exposure in vivo. Use of a T cell-deficient recipient expressing low amounts of Ag allowed for a massive expansion phase, followed by minimal deletion, resulting in mice with a large cohort of tolerant T cells. The persistence of the Ag was also critical, because low doses of Ag in a T cell-deficient host have been shown previously to lead to a memory state if the Ag is eliminated (26). The strong expansion phase (similar to what is seen for CD8$^+$ T cells in response to viral infection) (27) also resulted initially in extensive differentiation toward production of a wide variety of cytokines (28, 29). This allowed us to follow the onset of hyporesponsiveness, seen as a diminished potential to produce all of these cytokines over time. By 7–14 days after transfer, an equilibrium was reached in which cell expansion in vivo was minimal and cytokine production in vitro was reduced to 5–15% of its potential at day 1. Restimulation in vitro with increasing amounts of exogenous peptide also showed a shift in the proliferative dose-response curve and a desensitization for expression of the early activation markers CD69 and CD25. The latter was detected in a single cell assay (Fig. 3) and demonstrated that at least 50–70% of the cells were being affected. This adaptation to chronic Ag exposure appeared to be in the T cell population itself and not due to changes in the Ag presentation environment of the host, because a fresh cohort of naive T cells injected into an adapted host expanded normally. This experiment also ruled out active suppression as a mechanism. The hyporesponsive state was not caused by TCR down-regulation and persisted in vivo for months. We have also observed this state if the T cells were transferred into an irradiated mPCC host containing normal T cells, although the expansion phase in this case is not as great (data not shown). The hyporesponsive state was reversible if the T cells were transferred to a second CD3$\epsilon^{-/-}$ host not expressing the Ag. At least part of this reversal took place in the absence of proliferation, suggesting that the original mechanism for the limitation in cytokine production was not solely the loss of a subset of cells by a deletional or migrational process. Finally, in one preliminary experiment, stimulation of these second hosts at day 21 with 10 μg of the superantigen staphylococcal enterotoxin A demonstrated that the adapted cells were proliferatively tolerant in vivo. Expansion of the CD4$^+$ T cell population was 4.5-fold less in the Ag-bearing mPCC-CD3$\epsilon^{-/-}$ host than in a CD3$\epsilon^{-/-}$ host lacking the Ag. The latter expansion (18-fold) was equivalent to that seen for a control naive T cell population transferred into a CD3$\epsilon^{-/-}$ host under the same conditions (16-fold).

One of the most surprising findings in our experiments was the enhancement of the hyporesponsive state on retransfer of the cells into a second, T cell-deficient, Ag-bearing host. The cells initially appeared to partially recover from the tolerant state, both in terms of proliferation and cytokine production, but then they gradually lost sensitivity and eventually achieved a deeper level of unresponsiveness. This more profoundly depressed state was not solely due to cell deletion or redistribution in the second host, because the recovered cells themselves clearly became more desensitized at later time points (Fig. 9, *A* and *C*). The possibility that T cells can reach different levels of tolerance has been suggested before by Ferber et al. (30). However, in their model this phenomenon was seen when the cells were exposed to different concentrations of Ag in a serial manner. In our model, the Ag presentation is the same on re-exposure. Thus, some other variable(s) must have allowed the retransferred cells to readjust their activation threshold.

Several possible mechanisms come to mind. The first is a change in cell density. Only 3 million T cells, of 20–40 million in the first host, were injected into the second host. As we showed in vitro a number of years ago (31) and has been more recently demonstrated in vivo for thymic selection (32) and peripheral expansion (33, 34), T cells of similar specificity can compete with one another for activation by a limited number of peptide/MHC complexes. Thus, the lower density of T cells created on retransfer of a small number of them into a second empty host would effectively allow each cell to experience a higher concentration of Ag. This might be adequate to activate the hyporesponsive T cells. This idea is supported by the control experiment in which we transferred the T cells back into a day 38 host filled with tolerant cells. Under these conditions, the CFSE-labeled donor cells did not proliferate. Note that this T cell competition argument did not seem to apply to the naive T cells when they were injected into a day 38 tolerant host (Fig. 7). We think this is because the naive T cells are more responsive than the tolerant cells (compare Fig. 1 with Fig. 8*A*), and thus more effective competitors for peptide/MHC complexes. Alternatively, they go to different niches for their stimulation. In contrast, the expansion of naive T cells can be affected by the presence of memory T cells (26).

But even if the initial response on retransfer is a consequence of a change in cell density, it is not obvious why this would lead to a deeper state of tolerance, as opposed to a re-equilibration at the original level, once the T cell numbers expanded again. One possible explanation is that the expansion in the second host never reaches the same population size achieved in the first host, because of the impaired responsiveness of the retransferred T cells. In our experiments, the difference in population sizes achieved was ~4-fold. In this scenario, the final differences in cell densities would lead to a difference in effective Ag concentrations, which in turn would lead to a difference in adjusted tolerance threshold levels.

An alternative possibility is that only the proliferative response is activated by the change in cell density, but that this response itself brings about the reprogramming. Thus, similar to the idea that the chromatin is opened up at cytokine loci by appropriate signaling during proliferative expansion (28, 29), a tolerogenic environment might only effectively signal the closing down of a cytokine locus during a proliferative cycle.

Another possible explanation for the threshold changes on retransfer is that the costimulatory environment of the tolerant host has actually been modified, but that this change only influences the adapted T cells and not naive T cells. A host that has undergone an immune response might contain many nonprofessional APC that bear up-regulated costimulatory molecules in its tissues, such as B7-1 and B7h, induced in response to inflammatory cytokines such as TNF-α (35, 36). In a scenario in which negative regulation (e.g., CTLA-4) is dominant in the adaptive phase of the immune response, the tolerant T cells would be kept in check in the first host by CTLA-4/B7-1 interactions (37), whereas a naive cohort might be unaffected (Fig. 7). However, transfer of the tolerant cells to a new host would abruptly change the environment to one in which B7-1 expression is low. This would break the negative regulation cycle, and allow the cells to proliferate until up-regulation of B7-1 and CTLA-4 expression could again establish negative regulation in the second host. However, the final difference in activation thresholds would still require some additional changes to take place during the proliferative expansion, e.g., induction of more B7-1 in the environment of the second host or a decrease in signaling potential in the adapted T cell, as discussed above. Furthermore, arguing against this model is a preliminary experiment in which we injected anti-CTLA-4 mAb into tolerant mice for 5 days and did not enhance proliferation in vivo or cytokine production in vitro (L. Chiodetti, N. Singh, and R. Schwartz, unpublished observations).

What is the basis for the tolerance process? The absence of evidence for immunoregulation in the in vivo mixing experiment, coupled with the hyporesponsiveness of the T cells in vitro when they are stimulated with fresh APC and optimal Ag doses, suggests that the tolerance process is intrinsic to the T cells themselves. The shifts in the CD25, CD69, and proliferative dose-response curves in vitro all suggest a desensitization process. This is supported by the shift in cytokine production curves seen in the retransfer experiments, although a loss of cytokine-producing cells also contributes to the tolerance. Although this is the first clear description of an in vivo desensitization tolerance mechanism for CD4$^+$ T cells, it appears to be comparable in some ways to the phenomenon of B cell clonal anergy described by Goodnow and colleagues (38). Their model also involves two Tg mice in which a monoclonal B cell population becomes hyporesponsive after transfer into an Ag-bearing host or when crossed to the Ag-bearing mouse. Biochemically, the B cells appeared to down-regulate their IgM receptors by 90% and to show a block in early tyrosine phosphorylation events. The cells persisted in this state for a long time provided that there were no other normal B cells around, and the process was reversible on transfer to a host that did not express the Ag. In our adaptive T cell model, receptor down-modulation does not appear to be a major component of the tolerance process. Rather, preliminary data suggest a 2-fold inhibition in the ability to activate (with plate-bound anti-TCR) the early response kinases of purified CD4$^+$ T cells from the tolerant mice. This supports the idea of an adaptive process in which the T cell intrinsically alters its threshold for responding to signals through the TCR. One mechanism by which the T cells could enter such an altered state is through the expression of CTLA-4 (39, 40). This molecule is up-regulated following CD4$^+$ T cell activation, and inhibits TCR signaling by bringing the phosphatase SHP-2 into the receptor activation complex (41). Another is through the negative feedback recruitment of SHP-1 to the TCR activation complex following TCR signaling (42).

No matter what the nature of the intrinsic tolerant state turns out to be, the ability of this mechanism to achieve variable levels of unresponsiveness in the presence of a constant amount of Ag presentation is still a new and surprising finding. This adaptive property emphasizes the ability of the responding T cell to set different thresholds for activation depending on multiple cues from the environment. Constant environments seem to be tolerated, whereas changing environments elicit a reaction. A similar notion of individual T cells adjusting their response potential has been proposed to explain positive selection to agonist peptides in the thymus (43). To us, this type of adaptive tolerance model is most consistent with the theoretical thinking of Grossman and Paul (44), in which multiple environmental cues are integrated to determine the response threshold for T cell reactivation following Ag exposure.

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