The Strength of Persistent Antigenic Stimulation Modulates Adaptive Tolerance in Peripheral CD4$^+$ T Cells

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Abstract

The quantitative adaptation of receptor thresholds allows cells to tailor their responses to changes in ambient ligand concentration in many biological systems. Such a cell-intrinsic calibration of T cell receptor (TCR) sensitivity could be involved in regulating responses to autoantigens, but this has never been demonstrated for peripheral T cells. We examined the ability of monoclonal naive T cells to modulate their responsiveness differentially after exposure to fourfold different levels of persistent antigen stimulation in vivo. T cells expanded and entered a tolerant state with different kinetics in response to the two levels of stimulation, but eventually adjusted to a similar slow rate of turnover. In vivo restimulation revealed a greater impairment in the proliferative ability of T cells resident in a higher antigen presentation environment. We also observed subtle differences in TCR signaling and in vitro cytokine production consistent with differential adaptation. Unexpectedly, the system failed to similarly compensate to the persistent stimulus in vivo at the level of CD69 expression and actin polymerization. This greater responsiveness of T cells residing in a host with a lower level of antigen presentation allows us to demonstrate for the first time an intrinsic tuning process in mature T lymphocytes, albeit one more complex than current theories predict.

Key words: tuning • adaptation • CD69 • F-actin • anergy

Introduction

Cellular adaptation to environmental stimuli regulates a variety of biological responses ranging from chemotaxis in bacteria to visual perception in the eye. Many receptor systems capable of adapting their responsiveness are concerned with rapid changes in the concentration of extracellular ligand and use the steady state levels to generate appropriate negative feedback that effectively shuts down signal transduction in the absence of a change. The delay in the generation of such feedback also extends the duration of the response, thereby allowing the flexible design of cellular response states over a broad range of ligand concentrations.

In the innate immune system, quantitative receptor adaptation regulates neutrophil chemotaxis (1), macrophage activation by endotoxin (2), and possibly the recognition of self-MHC by NK cells (3). Developing T cells in the thymus undergo changes in the kinds of molecules mobilized to the TCR signaling machinery (4) and in the expression of coreceptors like CD5 (5) that allow the mature cells to show reduced responsiveness to a similar stimulus in the periphery (6). These changes effect a "tuning" of the TCR, ostensibly toward positively selecting ligands in the thymus, and help set the activation threshold for naive TCRs in the periphery (7–9) such that T cells in the periphery should only make a response if the level of antigenic stimulus exceeds this activation threshold.

The potential for individual mature T cells to modulate their activation thresholds in dynamic conversation with ambient antigenic stimulation is predicted by the tunable activation threshold (TAT) model of Grossman and Paul (10, 11). In this model, naive T cells maintain tolerance to self-MHC–peptide complexes by tuning their TCR signaling to match the persistent stimulus. A rapid change in the strength of stimulus initiates a response in these T cells but persistence of the new level of stimulus allows them to readjust their activation thresholds accordingly. The existence of such cell-intrinsic tunability of activation thresholds in indi-

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Abbreviations used in this paper: CFSE, 5,6-carboxy-fluorescein succinimidyl ester; HEL, hen egg lysozyme; MFI, mean fluorescence intensity; PCC, pigeon cytochrome c; TAT, tunable activation threshold.

vidual mature T cells has not yet been demonstrated experimentally. It has, in contrast, recently been reported that naive T cells removed from the presence of self-MHC interactions in vivo show a rapid loss of responsiveness (12).

Peripheral T cells exposed to tolerogenic forms of soluble or persistent self-antigen enter a state of unresponsiveness that significantly reduces their ability to make cytokines or proliferate (13–15). This state, called adaptive tolerance, is also accompanied by reduced transduction of various TCR-mediated signals (16). Furthermore, in contrast to T cells rendered clonally anergic (17), both monoclonal (18) and polyclonal (19) T cells adaptively tolerant to persistent self-antigens in vivo are able to recover responsiveness upon removal of the antigenic stimulus. The demonstration of such reversible states suggests that peripheral T cells can dynamically move between at least two extremes of responsiveness (on or off) depending on the presence or absence of persistent stimulation.

Application of the TAT model to the phenomenon of adaptive tolerance phenomena allows for a mechanism by which mature peripheral T cells could continue to assess their potential for autoreactivity and subsequently alter their responsiveness using continuous feedback from the environment. Self-reactive T cells could thereby downmodulate potentially autoimmune responses and yet continue to maintain a diverse repertoire capable of responding to a stronger stimulus from dangerous pathogens. Although there is some evidence that polyclonal repertoires of T cells may undergo selection for affinity during the course of an immune response (20, 21), affinity maturation by selection for individual clonotypes in a population with divergent TCRs does not address the possibility that activation thresholds might also be tuned at the level of individual T cells. Neither can this be addressed by studies using TCR transgenic populations from mice capable of endogenous TCR rearrangements, because of the high probability of expressing dual receptors. Therefore, the ability of monoclonal T cells to dynamically exist in multiple states of responsiveness coordinate with the level of ambient stimulus is yet to be demonstrated. The existence of such a cell-intrinsic adaptation process is a critical prediction of the Grossman and Paul model.

We adoptively transferred a monoclonal population of naive TCR transgenic T cells into two recipient mice presenting different persistent levels of the cognate antigen to ask if T cell tolerance in vivo demonstrates intrinsic adaptation to different ambient levels of stimulation. Consistent with the predictions of the TAT model, adaptively tolerized T cells resident in an environment with a higher level of antigen presentation showed a lower level of proliferation, cytokine production, and TCR signaling compared with similar T cells resident in an environment with a lower level of antigen presentation. Interestingly, T cell adaptation to the lower stimulus was kinetically slower and also less stable. Furthermore, the tuning was not a simple recruitment of negative feedback to prevent all responses to the stimulus, rather, it was manifested by the control of post-expansion turnover in vivo. Other markers of in vivo activation such as CD69 expression and actin polymerization were not compensated to equal levels, but in fact displayed greater responsiveness to the ambient stimulus of the low environment. These observations demonstrate that mature peripheral $CD4^+$ T cells undergo cell-intrinsic adaptation in response to persistent antigenic stimulation, albeit in a manner more complex than predicted by current theoretical models.

Materials and Methods

Mice. All mice were backcrossed onto the B10.A/SgSnAi background and bred at the NIAID contract facility at Taconic Farms, an American Association for Accreditation of Laboratory Animal Care–accredited specific pathogen-free barrier. The pigeon cytochrome c (PCC) 81–104-specific 5C.C7 TCR transgenic $RAG2^{-/-}$ mouse as well as B10.A,$CD3\varepsilon^{-/-}$, and mPCC(R0),$CD3\varepsilon^{-/-}$ mice have been described (18). The ePCC (SPK) transgenic created by Oehen et al. (22) expresses PCC under the control of the MHC-I promoter and Ig enhancer elements identical to the mPCC transgene. However, this construct lacks the neuraminidase membrane-targeting domain and therefore targets to the mitochondria. We bred the BALB/c,ePCC mouse (Jackson ImmunoResearch Laboratories) onto the B10.A,$CD3\varepsilon^{-/-}$ background and used them at N5 for our experiments. B10.BR,$3A9^{+/-}$ TCR transgenic mice were generated by Ho et al. (23) and provided by I. Gery (National Eye Institute of the NIH, Bethesda, MD).

T Cell Isolation, 5,6-Carboxy-Fluorescein Succinimidyl Ester (CFSE) Labeling, and Adoptive Transfer. Naive T cells used for adoptive transfer were obtained by pooling the cervical, axillary, brachial, inguinal, mesenteric, periaortic, and pancreatic lymph nodes of B10.A,5C.C7(Tg) $RAG2^{-/-}$ mice. Typically this yields 90–93% $CD4^+$ T cells and no further purification was performed. $3–5 \times 10^6$ cells were injected i.v. into recipient mice. For tracking in vivo division, cells were labeled with 2 μM CFSE (Molecular Probes) in PBS plus 0.5% FCS at 37°C for 15 min. Single cell suspensions from lymph nodes and spleens of recipients were stained with anti–Vβ3-PE (KJ25; BD Biosciences) and anti–CD4-TC (Caltag) to enumerate the percentage of T cells. T cells were depleted of recipient origin cells by two rounds of negative selection with dynabeads (Dynal) and antibodies to B220 (RA3-6B2), CD11b (M1/70), $I\text{-}E^k$ (17.3.3S), and CD8 (53-6.7; BD Biosciences). T cells for retransfer were derived from recipient mice that received unlabeled naive T cells and were labeled with CFSE as described above after the enrichment process. Surface expression of CD69 (H1.2F3), CD25 (PC61), CD5 (53-7.3), or CD44 (IM7) was examined with PE-labeled antibodies (BD Biosciences).

For intracellular F-actin staining, $1–2 \times 10^6$ freshly isolated cells from recipients were stained for surface TCR/CD4 before fixing and permeabilization using the Fix-Perm kit (Caltag) according to the manufacturer's instructions. Alexa 546–coupled phalloidin (Molecular Probes) was used to stain processed cells for 30 min on ice.

In Vitro T Cell Proliferation Assays and Cytokine Measurement. In vitro responsiveness of T cells to HPLC-purified PCC 81–104 peptide (Bachem) was routinely assayed using 10^4 T cells cocultured with half-log dilutions of the peptide and 5×10^5 irradiated (3,000 rads) B10.A splenocytes in a 1:1 mix of RPMI 1640 and EHAA (Biofluids Inc.) plus 10% FCS, 4 mM glutamine, 200 μg/ml penicillin, 200 μg/ml streptomycin, 25 μg/ml gentamicin, and 50 μM 2-ME. For anti-CD3 responses, 96-well flat-bottom

polystyrene plates (Corning Inc.) were coated with 2C11 (BD Biosciences) in carbonate buffer, pH 9.5, for 4 h at 37°C or overnight at 4°C. Plates were washed with culture medium and dried briefly before seeding with 10^4 T cells and anti-CD28 ascites (diluted 1:5,000).

After 48 h, 100 μl of the supernatant was removed for measuring secreted cytokines and remaining cells were cultured for an additional 20–24 h with 1 μCi per well of tritiated thymidine (Amersham Biosciences). Cells were harvested using a Brandel 96-well harvester and incorporated tritium was counted using a Wallac Trilux 1450 scintillation counter. All cytokine measurements used the Quantikine M kits (R&D Systems) according to the manufacturer's instructions.

For quantitating APC function in vitro, splenocytes were used as mixed populations or subsetted by sorting at the NIAID flow facility (to >98% purity) and inactivated by 3,000 rads irradiation (non–B cells) or 50 μg/ml Mitomycin C (B cell fractions).

A population of in vitro–generated preactivated T cells was used as a sensitive tester population to measure the antigen presentation by ex vivo–isolated mPCC and ePCC spleen cells. These cells were generated by stimulating 5C.C7,RAG2$^{-/-}$ lymphocytes with 1 μM PCC and irradiated B10.A splenocytes for 3–4 d. The stimulated T cells were harvested and separated from dead cells by layering on Lympholyte M (Accurate Chem). Live cells were "rested" by culturing in complete medium supplemented with 10 units/ml IL-2 for at least 7 d. Similar hen egg lysozyme (HEL)-specific 3A9 T cells were generated by two rounds of amplification with 1 μg/ml HEL presented first by B10.BR and subsequently by B10.A splenocytes.

Analysis of In Vivo Proliferation by CFSE Staining. Dilution of CFSE was quantitated as previously described (24). Areas under histogram peaks calculated using BD CELLQuest™ software was used to enumerate the number of cells in each division (i) at each time point. This number was divided by 2^i to derive numbers of input cells contributing to the peak. Total summation of such cohorts was used to normalize each peak as a percentage and fitted to a Gaussian distribution. The mean of that distribution was estimated and used as the mean number of divisions (*n*) of the population at that time. Values of *n* at multiple time points (t) were fitted to a linear regression and the rate of division (slope) and lag time were (value of t at $n = 1$) derived. This form of analysis was robust when applied to naive T cells ($R^2 = 0.95$–0.99 in the Gaussian fit), whereas antigen-experienced T cells showed significant variability between experiments ($R^2 = 0.48$–0.99). Part of this was due to the heterogeneity in the size and labeling of the antigen-experienced population.

BrdUrd Incorporation In Vivo. Turnover in vivo was measured by injecting 0.8 mg BrdUrd (Sigma-Aldrich) in PBS i.p. once every 12 or 24 h. Intracellular staining for BrdUrd used the BrDU Flow kit (BD Biosciences) according to the manufacturer's instructions.

Phosphorylation of ERK. 1–2 × 10^6 purified T cells were stimulated on 24-well plates coated with 10 μg/ml anti-CD3 in PBS at 37°C. T cells (in 50 μl volume) were stimulated for 1–15 min and the plate was quick frozen by floating on liquid nitrogen. Cell lysates were freeze thawed in 2X SDS sample buffer (Bio-Rad Laboratories) supplemented with 1 μM 2-ME and 10 μM Na_2VaO (Fisher Scientific). Lysates were boiled for 10 min, sonicated, and 45 μl were separated on an 8–16% SDS-PAGE gel (Bio-Rad Laboratories) for transfer to PVDF membranes (Hybond; Amersham Biosciences). Membranes were blocked with 5% Blotto (Santa Cruz Biotechnology, Inc.) and probed with anti-phospho ERK mAb (Cell Signaling or Santa Cruz Biotechnology, Inc.), followed by biotinylated anti–mouse antibody (Santa Cruz Biotechnology, Inc.) and Avidin-Biotin AP (Vector Laboratories). The bound AP was detected using the ECF reagent (Amersham Biosciences). Blots were stripped of the bound antibody and reprobed with anti-ERK (Upstate Biotechnology) and biotinylated anti–rabbit IgG (Santa Cruz Biotechnology, Inc.). The amount of pERK signal was detected on a Molecular Dynamics PhosphorImager and is expressed as a percentage of the total ERK signal from the same lane.

Online Supplemental Material. T cells adapted to PCC-lo or PCC-hi levels of antigen presentation were recovered 34 d after the transfer, purified, and stimulated with plate-bound anti-CD3 and soluble anti-CD28. The amount of IL-4 (Fig. S1 a), IL-10 (Fig. S1 b), and TNF α (Fig. S1 c) secreted is shown in Fig. S1. The expression of activation markers CD25 (Fig. S2 a), CD44 (Fig. S2 b), or CD5 (Fig. S2 d) and the levels of the TCR (Fig. S2 c) on the T cell resident in the PCC-hi or PCC-lo environments

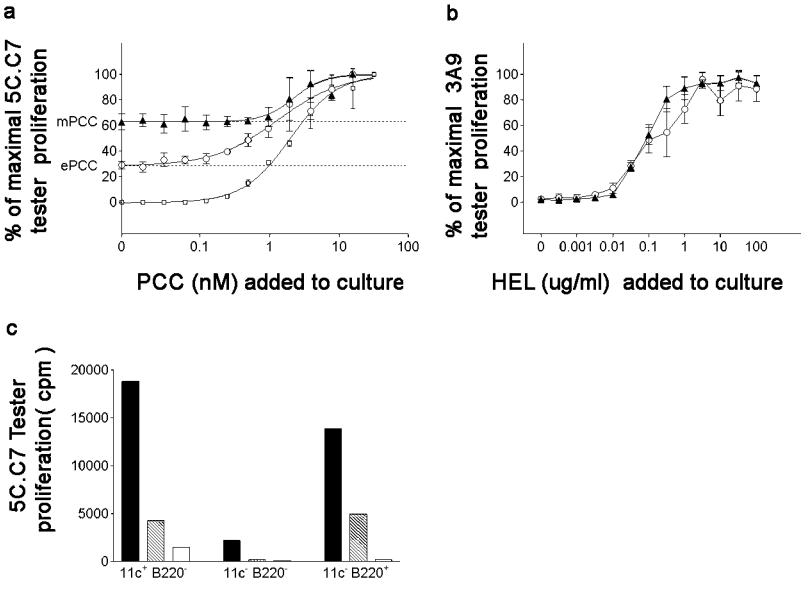

Figure 1. In vitro antigen presentation by mPCC splenocytes is greater than that by ePCC splenocytes. (a) Functional presentation of the transgene-derived antigen by mPCC,CD3ε$^{-/-}$ (▲) and ePCC,CD3ε$^{-/-}$ (○) APCs to T cells in vitro was quantitated by comparing the proliferation of PCC-specific tester T cells to fresh splenocytes from either transgenic or antigen-deficient CD3ε$^{-/-}$ (□) mice. Tester T cells used were from a rested, in vitro–preactivated population of 5C.C7 T cells, which are extremely sensitive to relatively subtle variations in antigen presentation. Dotted lines from the y axis denote basal proliferation elicited by each type of APC estimated from a curve fit of the data. (b) Proliferation of 3A9 T cells to HEL presented by splenocytes as in (a) shows that the presentation differences are specific to PCC. (c) Presentation of antigen by the two transgenics is not differentially compartmentalized in splenic APCs. Sorted CD11c$^+$ B220$^-$, CD11c$^-$ B220$^-$, or B220$^+$ cells freshly isolated from the mPCC,CD3ε$^{-/-}$ (solid bars), ePCC,CD3ε$^{-/-}$ (hatched bars), or CD3ε$^{-/-}$ mice (open bars) were used to stimulate tester T cells in vitro.

for different periods of time (after adoptive transfer) is shown in Fig. S2. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20030913/DC1.

Results

Differential In Vitro Presentation of PCC by Splenocytes from mPCC and ePCC Transgenic Mice. To generate host environments expressing different amounts of the same "self"-antigen, we introduced the homozygous CD3$\varepsilon^{-/-}$ mutation into two PCC transgenic mice (mPCC and ePCC) originally created by Oehen et al. (22). Quantitation by Northern hybridization revealed that splenocytes from the CD3$\varepsilon^{-/-}$ mice homozygous for the ePCC transgene expressed fivefold lower levels of the antigen transcript than those homozygous for mPCC (unpublished data), confirming earlier observations in T cell–sufficient hosts (22). For the purposes of this study it was critical to test if the differences in message levels were also reflected in differential presentation of processed antigen to T cells by APCs. We assayed this by using freshly isolated splenocytes from each host to stimulate a population of previously activated PCC-specific tester T cells, generated as described in Materials and Methods (Fig. 1 a). In the absence of added PCC peptide, the mPCC,CD3$\varepsilon^{-/-}$ splenocytes elicited ~63% of maximal tester cell proliferation. In contrast, the ePCC, CD3$\varepsilon^{-/-}$ splenocytes elicited only 28%. The intersection of these basal proliferation values on the antigen-induced proliferative dose response curve obtained with CD3$\varepsilon^{-/-}$ splenocytes not expressing PCC (Fig. 1 a, horizontal lines), shows that presentation by mPCC,CD3$\varepsilon^{-/-}$ splenocytes is equivalent to a fourfold (average difference of 4.1-fold, n = 4) increase in antigen concentration over that presented by ePCC,CD3$\varepsilon^{-/-}$ splenocytes.

Fractionation of the splenocytes from the two hosts into CD11c$^+$ B220$^-$ (dendritic cells), CD11c$^-$ B220$^-$ (macrophages), or B220$^+$ (B cells) by FACS® sorting to >98% purity, did not reveal any differential compartmentation of the antigen in the two hosts (Fig. 1 c). We also ruled out the possibility that the antigen in these two mice was expressed differentially in other tissue locations by using lysates from multiple tissues as a source of antigen to stimulate previously activated T cells in vitro. Only lysates from spleen and lymph nodes resulted in significant stimulation and in both cases mPCC,CD3$\varepsilon^{-/-}$ tissues yielded more stimulation than ePCC,CD3$\varepsilon^{-/-}$ tissues (unpublished data). Finally, APCs from the two mice did not show a similar difference in their ability to stimulate an HEL-specific cell line derived from 3A9 TCR transgenic mice (Fig. 1 b), arguing for antigen specificity in the differential presentation. Therefore, we refer to the mPCC,CD3$\varepsilon^{-/-}$ mice as PCC-hi and the ePCC,CD3$\varepsilon^{-/-}$ mice as PCC-lo recipients for the rest of this report.

In Vivo Proliferation of Monoclonal T Cells to Different Levels of Persistent Antigen Stimulation. A monoclonal population of >92% pure naive CD4$^+$ T cells specific for the PCC 81–104 peptide, isolated from the lymph nodes of B10.A,5C.C7,RAG2$^{-/-}$ TCR transgenic mice, was i.v. injected into the PCC-hi or PCC-lo antigen transgenic hosts. Within 2 h after adoptive transfer, 10–20% of the injected 5C.C7 T cells populate the nodes and spleen of either the PCC-hi or PCC-lo hosts. This number was similar in the two recipients and did not significantly increase over the next 24 h. T cells in either host started proliferating in vivo between days 1 and 2 after transfer (Fig. 2 a). The initial burst of expansion to the PCC-hi stimulus resulted in a 30–70-fold increase in recovery of T

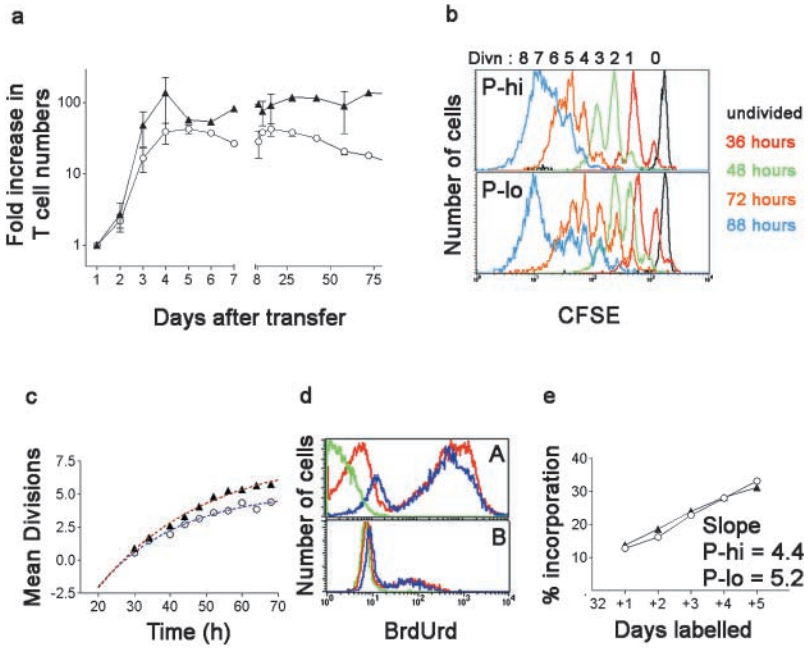

Figure 2. Naive T cells expand differentially in the two environments but eventually achieve similar turnover rates. (a) Naive 5C.C7,RAG2$^{-/-}$ T cells transferred into the PCC-hi (▲) or PCC-lo (○) recipients were recovered after various days. The absolute number of recovered T cells on each day was normalized to that on day 1 in each group. Three experiments are averaged on days 1–5 and for subsequent time points showing error bars. (b and c) Different levels of persistent antigen induce T cell cycling with the same lag time, but affect the subsequent rate of proliferation. Dilution of CFSE in the T cells recovered from PCC-hi or PCC-lo hosts was analyzed using the Gett-Hodgkins method to calculate the cell division profile over the first 68 h. Overlay of dotted lines in (c) represents the raw data fitted to an exponential rise to the max function. The best fit parameters (R^2 = 0.97–0.99) for the PCC-hi and PCC-lo data are y0 = −11.9 ± 3.3 and −12.2 ± 5.2, a = 19.8 ± 2.5 and 17.5 ± 4.7, and b = 0.966 ± 0.008 and 0.957 ± 0.011, respectively. (d and e) Proliferative expansion is contained earlier in the higher stimulus environment, but is eventually modulated to similar extents in the two hosts. (d) BrdUrd incorporation in the PCC-hi (red lines) or PCC-lo (blue lines) resident T cells was measured after daily injections of BrdUrd between days 7 to 11 (A) or 32 to 36 (B). Green lines represent negative controls. (e) Incremental BrdUrd incorporation over the 5 d of labeling from days 32 to 36.

cells by the third day. Subsequently, the rate of net expansion slowed down, yielding one more population doubling by day 4 and accounting for a 75–150-fold total expansion of the transferred T cells. As previously reported (18), the expansion phase in the PCC-hi host is followed by a small decline in recovery (between days 4 and 7) and a subsequent steady state in which the population size increases only marginally out to 150 d. The initial expansion in the PCC-lo host was similar to that in the PCC-hi environment. By the third day after transfer, however, it was significantly slower and reached a peak of only 30–50-fold by day 4. Consistent with this reduced proliferative burst, the subsequent steady state T cell recoveries from the PCC-lo recipients were consistently two- to fourfold lower than the PCC-hi host (mean of 2.82-fold; $n = 20$; $P < 0.0001$). This lower plateau occurs despite the fact that the T cells do not go through a significant decline between days 4 and 12 in the PCC-lo host. However, at very late time points (beyond 70 d), there appears to be a sizeable loss of cells in the PCC-lo host (unpublished data). Similar transfers into antigen-deficient, T-deficient environments did not induce proliferation for up to 6 d and the subsequent slow "lymphopenia-driven" proliferation resulted in only a four- to fivefold net expansion as late as 2 mo after transfer (18).

To better quantitate the initial rate of expansion of the naive 5C.C7 T cells in the two transgenic hosts, we analyzed the dilution of the CFSE dye in T cells recovered at 4–6-h intervals between 24 and 72 h after adoptive transfer (Fig. 2 b). The mean division number was calculated and plotted against the time of recovery (Fig. 2 c). This form of analysis allowed us to mathematically model the initiation of cycling and the subsequent rate of division of the transferred T cells. The theoretically estimated lag time for entry into division was the same in both the PCC-hi and PCC-lo hosts. After entry into cycle, the analysis revealed an extremely rapid period of division in both hosts (Fig. 2, b and c). In the PCC-hi host, the mean doubling time was 5.9 h between 30 and 52 h. This initial pace was slightly faster than in the PCC-lo host, where the mean doubling time was 7.2 h. For three separate experiments the average mean doubling time in the PCC-lo host (5.8 h) was 25% longer than in the PCC-hi host (4.7 h). This is consistent with the observed decrease in net expansion in the PCC-lo host by approximately one division at the end of 48 h. After 52–56 h, the T cells in both hosts lose their initial momentum and divide more slowly. A second linear fit of the data generated in the PCC-lo host from 56–72 h revealed that the T cells now divided once every 21.7 h (mean of two experiments) compared with 15.1 h in the PCC-hi host. This 43% increase in the doubling time in the PCC-lo host reduced the mean expansion at 72 h by approximately two divisions relative to the PCC-hi host. The proliferative behavior of the T cells at all of the time points through 68 h could also be modeled as an exponential rise to the max function [$y = y0 + a\,(1 - b^x)$] (Fig. 2 c, dotted lines). This mathematical analysis predicts that in contrast to reports concerning in vitro T cell proliferation (24, 25), the strength of persistent antigenic stimulus in vivo regulates the rate of cell cycle progression and not the time to first division. We further confirmed this observation by analyzing the percentage of naive T cells recruited to division at shorter intervals from 28 to 38 h. Analysis of seven such time points revealed no significant difference between T cells in the hi and lo hosts (mean ratio of 1.02; $P = 0.16$).

We were also able to estimate the loss of cells during the expansion phase by calculating the theoretical expansion of T cells from the CFSE profiles and comparing the actual numbers recovered at 72 h. Over three experiments, we could account for 78 ± 9% of the T cells in the PCC-hi and 82 ± 11% of the cells in the PCC-lo hosts, suggesting that there was minimal effect of cell death or migration in either host during the expansion phase between days 1–3 in vivo.

The in vivo turnover of cells after day 3 could not be reliably assayed by CFSE analysis due to poor resolution of the peaks after seven to eight divisions. Therefore, we evaluated the turnover by BrdUrd labeling (Fig. 2, d and e). Daily injections of BrdUrd between days 7 and 11 (5 d) in the PCC-hi host resulted in incorporation by 56% of the T cells. This level was substantially greater (79%) in the PCC-lo host (Fig. 2 d, A). Thus, although the initial rate of expansion was more rapid in the PCC-hi host, the turnover was also contained more rapidly in this environment, resulting in a transient reversal of the rate of proliferation in the two hosts. 32 d after transfer, however, a 5-d labeling (with twice daily injections of BrdUrd) resulted in comparable in vivo incorporation in the two T cell populations (31.2% in the PCC-hi host and 33.2% in the PCC-lo host; Fig. 2 d, B). 18 independent comparisons of BrdUrd incorporation over various labeling durations between days 22 to 76 yielded a mean PCC-lo/PCC-hi ratio of 1.02 (×/÷ 1.15). Finally, quantitation of the daily BrdUrd incorporation during the 5-d labeling between days 32 and 36 (Fig. 2 e) revealed a least squared fit slope of ~5% of the cells dividing every day in both hosts. Therefore, after the initial phase of rapid expansion was contained, the turnover of T cells in either environment was constrained to similar rates, consistent with adaptation of the T cells to the same level of proliferative responsiveness, despite persistent differences in the level of antigen presentation.

Differential Proliferative Responsiveness of PCC-hi– and PCC-lo–adapted T Cells In Vivo. The restriction of in vivo proliferation in both hosts is consistent with an adaptive process but could also be influenced by T cell density and other environmental influences. To compare the intrinsic in vivo proliferative potential of the two T cell populations, we retransferred 2–3 × 10^6 purified T cells into fresh PCC-hi hosts. As previously reported (18), PCC-hi–adapted T cells proliferated more slowly than naive T cells to this fresh challenge (Fig. 3 a), reaching a peak expansion of 5–8 × 10^6 cells (compared with 40–60 × 10^6 for naive T cells) by 4 d. Interestingly, as early as day 2, T cells recovered from the PCC-lo host seemed to expand faster than the PCC-hi–experienced T cells. Over the next 3 d, PCC-lo–experienced T cells expanded to a two- to threefold higher level (10–19 × 10^6 cells) than the PCC-hi–adapted

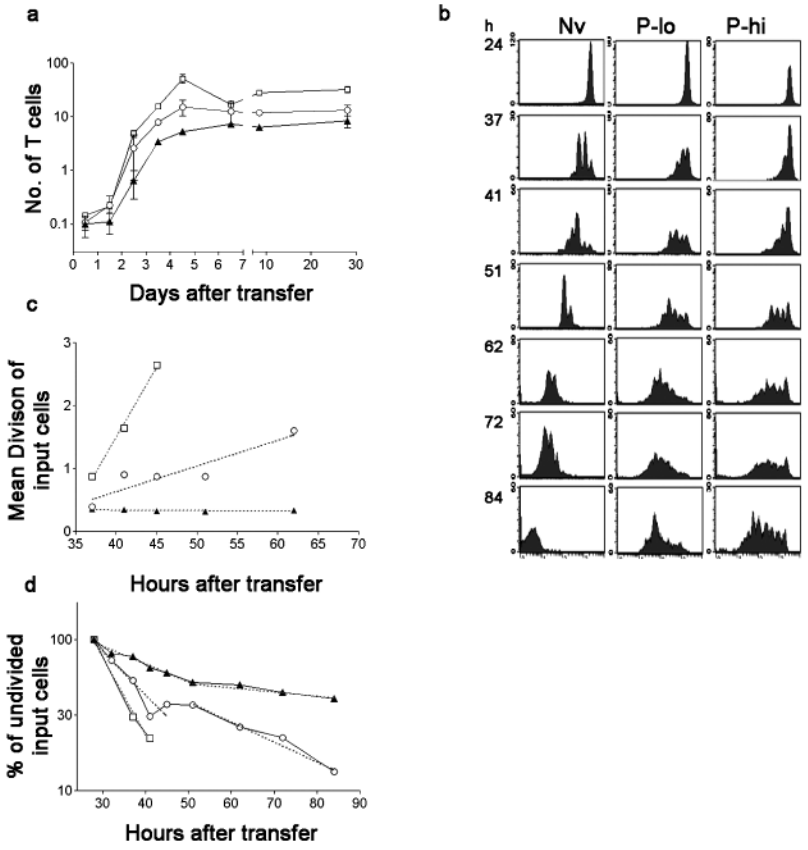

Figure 3. The quantitative impairment of in vivo responsiveness varies with the level of persistent antigen presentation experienced. (a) 5C.C7,RAG2$^{-/-}$ TCR transgenic cells resident for 22–28 d in the PCC-hi host (▲) proliferate poorer than those resident in the PCC-lo host (○) upon retransfer into a fresh PCC-hi environment. Both the adapted T cells are desensitized relative to naive T cells (□) establishing a hierarchy of adaptations in vivo. Data are averaged over three separate experiments. (b) The expansion of adapted T cells was monitored by CFSE dilution over short intervals of time after transfer of 28-d PCC-hi–adapted (P-hi), PCC-lo–adapted (P-lo), or naive (Nv) T cells into fresh PCC-hi mice. Numbers on the left represent the time points sampled in hours. (c) Quantitative analysis of the CFSE profiles in (b) by the Gett-Hodgkins method. (d) Initiation of cell cycling in the transferred T cells quantitated by the disappearance of the zero division CFSE peak reveals two phases of recruitment in the adapted T cells. Both phases, however, decay at greater rates for the PCC-lo–adapted T cells. Overlay of dotted lines on each curve represent the fitting of those parts of the data to a linear regression.

T cells, yet significantly lower than naive T cells. The difference in expansion resulted in a 2.09 ×/÷ 1.27-fold (n = 4; P = 0.006) higher plateau level for the PCC-lo–adapted T cells compared with the PCC-hi–adapted cells.

The differences in the proliferation of these adapted T cells was quantitated by following the dilution of CFSE in the dividing cells at short intervals of time between 24 and 72 h (Fig. 3 b). In an analysis of the CFSE peaks by the Gett-Hodgkins method (Fig. 3 c), naive T cells expanded initially with a doubling rate of 4.5 h (R^2 = 0.99). The PCC-lo–adapted T cells had an average doubling time of 24.5 h (R^2 = 0.85). Most of the T cells in the PCC-hi–adapted population did not divide in this early time interval, making it difficult to accurately determine the doubling time for this cohort. Therefore, we supplemented this form of analysis with a quantitation of the disappearance of the undivided CFSE peak over the first 84 h (Fig. 3 d). In this analysis, naive T cells divided very rapidly between 30 and 48 h, exiting the undivided pool at a rate of 5 ± 0.5% per hour (estimated from the slope of the linear regression overlay in Fig. 3 d). All the input cells had divided at least once by 45 h. In contrast, both tolerant populations displayed a biphasic decay pattern. In the first linear phase (dotted lines mark the two linear phases of the decay in Fig. 3 d), 35% of the PCC-hi–adapted T cells divided out of the input population at the rate of 1.2 ± 0.1% per hour. This initially dividing cohort was significantly larger (69%) in the PCC-lo–adapted T cells and their rate of entry into cycle was 2.4-fold faster (at 2.9 ± 0.6% per hour). Subsequently, a second phase of division was evident in both tolerant populations, but again the PCC-hi–adapted T cells proceeded at a slower rate (0.4% per hour) than the PCC-lo–adapted T cells (1.3% per hour). Complete removal of the undivided population took ~84–96 h for the PCC-lo–adapted T cells, whereas even at this time, undivided T cells were still evident in the PCC-hi–adapted cells. Thus, although both the adaptively tolerized populations display a complex mixture of proliferative behaviors, each component reveals about a threefold difference in responsiveness between the two experimental groups when confronted with an identical in vivo challenge. Such a gradation in the in vivo proliferative behavior is consistent with the existence of different thresholds for responding to a strong antigenic challenge by CD4$^+$ T cells adapted to different levels of persistent antigen presentation.

Strength of Stimulation Influences the Kinetics and Extent of Adaptive Tolerance. Adaptive tolerance in vivo does not result in a complete loss of in vitro T cell proliferation, but significantly reduces the ability of the cells to secrete a variety of cytokines in vitro (18). We compared this phenotype in T cells resident for various periods of time in either the PCC-lo or PCC-hi recipient. T cells were purified away from the host cells by negative selection (yielding a T cell population of between 70 to 90% purity) and stimulated in vitro with titrations of PCC 81–104 peptide on irradiated

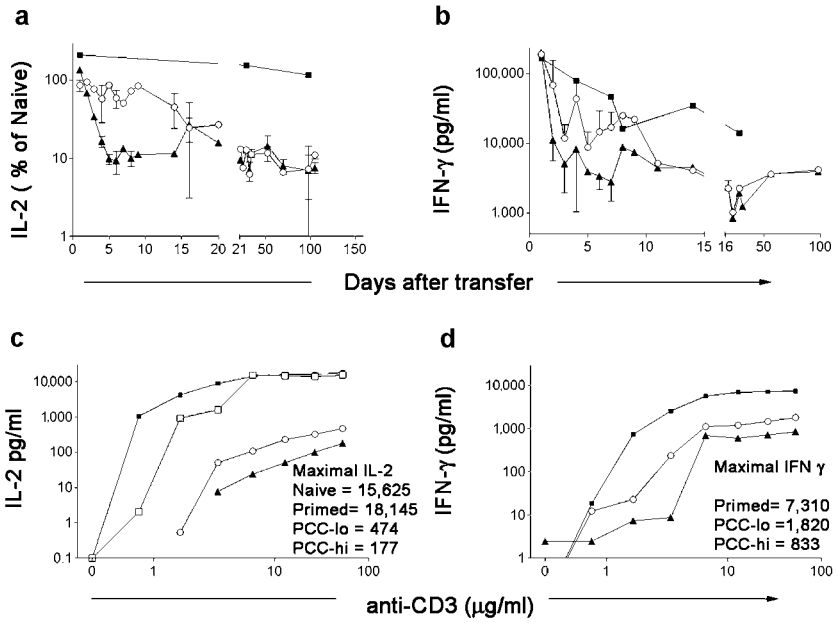

Figure 4. Adapted T cells down-regulate cytokine production with different kinetics and to different extents. (a and b) Maximal cytokine production after a 48-h APC plus peptide stimulation of 10^4 purified T cells recovered from the PCC-hi (▲), PCC-lo (○), or PCC plus LPS-primed (■) mice at different days after transfer reveals a faster down-regulation of IL-2 (a) and IFN-γ (b) production in the PCC-hi environment. (a) Maximal IL-2 production is expressed as a percentage of that produced by naive T cells. (b) IFN-γ production is expressed as absolute production in picograms per milliliter. Data points in (a) and (b) are averaged over two to five separate experiments for points shown with error bars. (c and d) After stable maximal down-regulation, a subtle difference in the responsiveness of PCC-hi– versus PCC-lo–adapted T cells can be revealed by stimulation with plate-bound anti-CD3 plus soluble CD28. Representative production of IL-2 (c) and IFN-γ (d) by T cells recovered 34 d after transfer. IL-2 production is compared with that by T cells from a naive 5C.C7,RAG2$^{-/-}$ TCR transgenic mice (□).

B10.A splenocytes (Fig. 4, a and b) or titrations of plate-bound anti-CD3 and soluble anti-CD28 (Fig. 4, c and d). Cytokine production from such stimulation assays were compared with naive 5C.C7 T cells or T cells previously primed in vivo with a single pulse of PCC plus LPS as described in Materials and Methods.

Upon stimulation with APC plus peptide or anti-CD3 plus anti-CD28, T cells resident in either antigen-bearing host for 20 d or longer consistently showed a dramatic down-regulation (~90%) of IL-2 secretion compared with naive 5C.C7 T cells or primed T cells (Fig. 4 a). Relative to primed T cells, the adapted cells also down-regulated IFN-γ production by up to 85% (Fig. 4 b). As previously reported (18), T cells that had expanded in an antigen-free, lymphopenic environment did not down-regulate cytokine production and behaved similarly to the primed T cells in our assays.

A kinetic comparison of the onset of the tolerance process in the PCC-hi and PCC-lo recipients revealed interesting differences. Down-regulation of IL-2 production in the PCC-hi host was completely achieved between days 4 and 5 (Fig. 4 a), whereas in the PCC-lo host it took much longer (16–20 d for the maximal effect). A similar phenomenon was observed for IFN-γ (Fig. 4 b). After a day of residence in either host, naive T cells were primed for IFN-γ production upon in vitro restimulation, although this initial priming was occasionally less efficient in the PCC-lo host. Residence for a period of 6–7 d in the PCC-hi host depleted 50–90% of the IFN-γ production and this loss was stably maintained for 100 d. In contrast, down-regulation took between 9–12 d to complete in the PCC-lo host, but was also maintained beyond 3 mo.

After completion of the down-regulation process in both environments, however, we could still detect subtle differences in the maximum cytokine production in vitro between the two adapted T cells, but only with the anti-CD3 plus anti-CD28 stimulus (Fig. 4, c and d). The differences in maximal IL-2 (mean difference of 2.08-fold in five experiments; $P = 0.0006$ in a paired two-tailed *t* test) and IFN-γ (mean difference of 1.9-fold in three experiments; $P = 0.01$) production between the PCC-lo– and PCC-hi–experienced T cells was consistent over multiple experiments. The cytokine down-regulation effect was global and affected IL-4, IL-10, and TNFα (Fig. S1, a, b, and c, available at http://www.jem.org/cgi/content/full/jem.20030913/DC1). Therefore, although the PCC-hi– and PCC-lo–adapted T cells were profoundly desensitized relative to naive or primed T cells, they also displayed subtle gradations in their in vitro responsiveness relative to each other. These data confirm the existence of a tuning process that is proportional to the strength of the ambient stimulus.

Quantitative Adaptation of TCR Signaling in the PCC-hi and PCC-lo Resident T Cells. The differences in anti-CD3– plus anti-CD28–elicited cytokine production between T cells adapted to the PCC-lo and PCC-hi environments suggested that signal transduction downstream of the TCR might have adjusted differently to the two levels of antigen presentation. To examine this possibility, we assayed the activation of the Ras-MAPK pathway in the PCC-lo– and PCC-hi–adapted T cells at the level of ERK phosphorylation. In the adaptive tolerance phase (day 13 in Fig. 5 a and day 28 in Fig. 5 b), PCC-hi–adapted T cells showed a 1.7-fold (Fig. 5 c) or 3.4-fold (Fig. 5 d) reduction in maximal ERK phosphorylation relative to naive T cells. In the same time frame, PCC-lo–adapted T cells showed only a 1.2-fold (Fig. 5 a) or 2.2-fold reduction (Fig. 5 b). Together with a third experiment, the PCC-hi–adapted cells showed a 1.3-fold greater mean reduction in maximal ERK phosphorylation relative to the PCC-lo–adapted T cells ($P = 0.004$). This differential down-regulation of

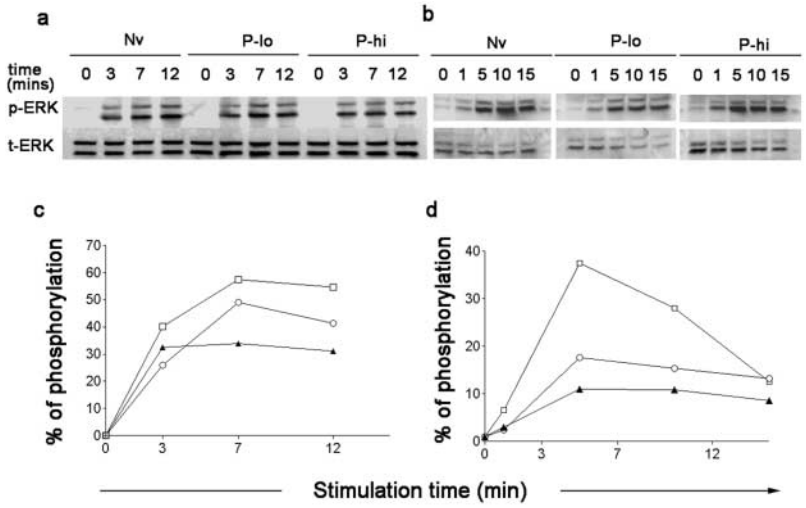

Figure 5. Biochemical alterations in adapted T cells. (a and b) TCR signal transduction assayed at the level of ERK phosphorylation is differentially affected in the PCC-hi–adapted (P-hi, ▲) versus PCC-lo–adapted (P-lo, ○) cells relative to naive T cells (Nv, □). T cells stimulated for various times on anti-CD3–coated plates and analyzed for phosphorylated ERK (p-ERK). Blots were stripped and reprobed for total ERK (t-ERK). (c and d) The percentage of phospho-ERK was quantitated using a PhosphorImager. Adapted samples were recovered 13 (a and c) or 28 d (b and d) after transfer.

TCR proximal signaling is likely to constitute at least part of the mechanism for the quantitative adaptation of T cell responses that we have observed.

A Second Phase of T Cell Activation to Persistent Antigen Stimulation Reveals Cell-intrinsic Differences in Adaptive Tolerance. The expression of activation markers during the response of T cells in vivo is a robust measure of single cell responses. In the presence of persistent antigen, activation of naive adoptively transferred T cells can be studied by the expression of activation markers in two phases: a potent early phase that is completed by 2–3 d, followed by a more subdued second phase after 10–14 d (18). We compared the two phases of activation after adoptive transfer of naive 5C.C7,RAG2$^{-/-}$ T cells into the PCC-hi and PCC-lo environments (Fig. 6). All of the recovered T cells in either host up-regulated CD69 with similar kinetics, reaching a peak expression between 6–12 h. However, the maximal level of expression (as indicated by the mean fluorescence intensity [MFI] of CD69 staining in Fig. 6 b) was about twofold higher in the PCC-hi host, consistent with a stronger stimulus. CD69 expression was barely detectable by 3 d of residence in both hosts. The IL-2 receptor α chain (CD25) was maximally

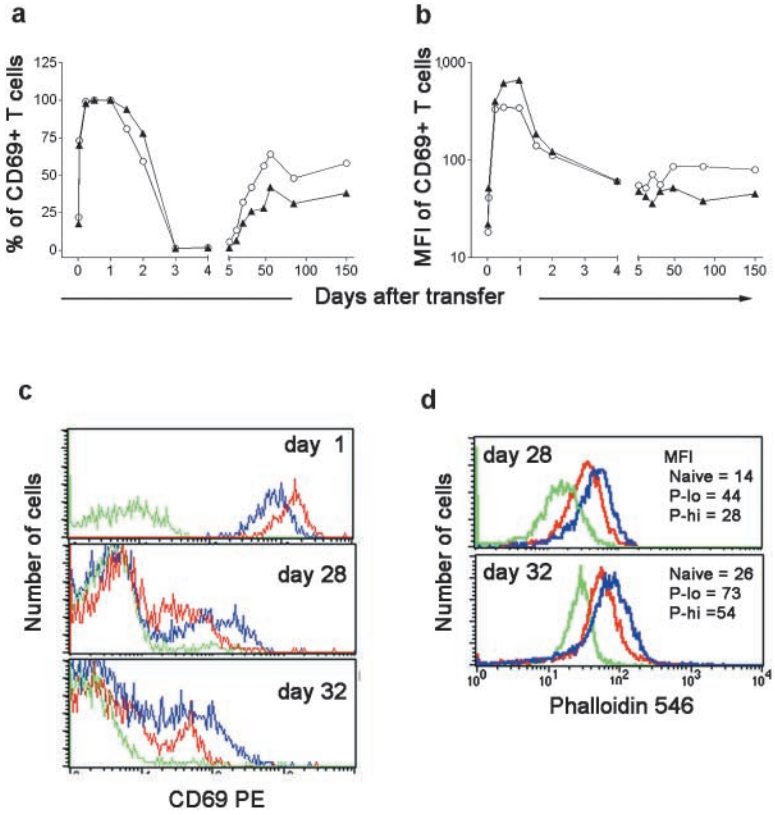

Figure 6. In vivo T cell reactivation during the adaptive tolerance phase reveals cell-intrinsic differences. (a–c) Surface expression of CD69 on naive 5C.C7,RAG2$^{-/-}$ T cells transferred into the PCC-hi (▲ or red lines) or the PCC-lo (○ or blue lines) recipients reveals an early phase of greater responsiveness in the PCC-hi host, followed by greater responsiveness in the PCC-lo host during the adaptive tolerance phase. (a) Percentage of cells expressing CD69 on various days after transfer and (b) the MFI of CD69 on the positive subset of cells on each day. Green lines represent naive 5C.C7 T cells. (d) T cells were stained for polymerized intracellular actin with Alexa 546–coupled phalloidin, ex vivo.

up-regulated within 12–24 h in both hosts and was not re-expressed after the second day (Fig. S2 a, available at http://www.jem.org/cgi/content/full/jem.20030913/DC1). All of the transferred T cells up-regulated CD44 by day 3 and remained $CD44^{hi}$ as long as 150 d past transfer (Fig. S2 b, available at http://www.jem.org/cgi/content/full/jem.20030913/DC1). Although the levels of TCR on the transferred T cells were comparable to naive T cells for up to 40 d, a significant down-regulation of the TCR was sometimes observed in the PCC-lo host past this time (Fig. S2 c, available at http://www.jem.org/cgi/content/full/jem.20030913/DC1). The surface expression of CD5 has been reported to influence TCR sensitivity (9) in naive T cells. Although CD5 levels were up-regulated in the adaptively tolerant T cells (relative to naive T cells), we did not observe a consistent difference in the expression of this marker between the PCC-hi– and PCC-lo–adapted T cells (Fig. S2 d, available at http://www.jem.org/cgi/content/full/jem.20030913/DC1).

Surprisingly, during the second phase of CD69 expression, 10–20% more cells in the PCC-lo host displayed the marker (Fig. 6 a) with a higher MFI (Fig. 6, b and c). The higher MFI suggests greater transduction of the TCR signal by the PCC-lo resident T cells despite the actual ambient stimulus being lower than in the PCC-hi host. To corroborate this conclusion, we examined an independent activation marker for in vivo TCR signal transduction. TCR signals are routed through Vav to polymerize actin and establish the T cell synapse (26). T cells recovered in the adaptive phase from PCC-lo recipients showed a 1.46-fold ($\times/\div 1.11$; $n = 3$; $P = 0.01$) higher level of intracellular polymerized actin ex vivo than those resident in the PCC-hi host, as measured by the binding of fluorescent phalloidin (Fig. 6 d). These F-actin levels were uniformly up-regulated in all of the PCC-lo–adapted T cells. Thus, although the first phase of naive T cell activation was greater in the PCC-hi host, at later times PCC-lo resident T cells evidence greater responsiveness to the ambient stimulation. In conjugation with our previous demonstration of reversibility of this adaptively tolerant state (18), these data strongly argue that the tuning process is cell intrinsic.

Discussion

The TAT model is a theoretical framework for the prediction of how mature peripheral T cells might dynamically adapt their responsiveness in vivo. Naive T cells are postulated to exist in a tuned state, i.e., in equilibrium with the level of self-MHC–peptide complexes, and can proliferate only if the ambient stimulus rapidly exceeds that of the steady state. Consistent with this assumption, our transfer of naive T cells from a PCC-free environment to one expressing PCC elicits a proliferative burst. During the initial phases of this response, the antigen presentation environment in the host undergoes subtle alterations (unpublished data) but returns to its original steady state within 4–5 d as measured by the ability of a labeled second cohort of naive T cells to proliferate in this environment (18). In this new steady state of persistent antigen presentation, the original T cells enter a form of hyporesponsiveness that appears to last from several weeks to months. Upon removal from this steady state and transfer into an antigen-free, T cell–deficient host, the T cells gradually recover their responsiveness (18). The TAT model would suggest that this reversible hyporesponsiveness results from a tunability of TCR activation thresholds, dictated by the extent of persistent stimulation available in the environment. Accordingly, a fourfold difference in persistent antigen presentation should also result in different adaptive states in the transferred T cells. Such a difference was observed in a comparison of the ability of the recovered T cells to proliferate in vivo as well as to signal and produce cytokines upon direct stimulation of the TCR complex in vitro. Sustained exposure to an environment expressing more antigen-specific APC function (PCC-hi) resulted in greater refractoriness to T cell reactivation. Therefore, this basic set of observations is compatible with the original predictions of the TAT model.

One clear functional consequence of the adaptive process in this system is to adjust the in vivo turnover of T cells to similar levels (Fig. 2 d) despite a fourfold difference in stimulation strengths. Proliferation is an endpoint of a cellular calculus integrating the strength and duration of TCR occupancy and costimulatory signaling at the nuclear level (27). Therefore, the effective proliferative signal perceived by T cells, showing the same turnover in the high and low stimulus environments, must be similar. Because the actual antigen presentation in the two mice are different, the integrated signal can only be equivalent if one or more of the TCR signaling cascades are more strongly inhibited in the PCC-hi host. We have shown that this is indeed true in one pathway: that of Ras-MAPK at the level of ERK phosphorylation. The actual nature of the "tuning machinery" might involve the recruitment of proportional negative feedback into multiple pathways downstream of the TCR (28 and unpublished data). Yet, the adaptive states in the two hosts display some differences that are inconsistent with a simple linear compensation of signal strength by negative feedback. The initial expansion of naive T cells in the two hosts stabilizes at two- to fourfold different numbers in the adaptive phase. Negative feedback that is elicited in linear correspondence to the strength of stimulus might have been expected to enforce a greater slowdown in the PCC-hi environment relative to the PCC-lo environment, effectively achieving similar plateau levels in the two hosts. In our system, however, the stopping of expansion actually precedes the achievement of complete tolerance (at least in the PCC-lo environment) as measured by cytokine down-regulation and in vivo proliferation. It is therefore arguable that the adaptive tolerance measured at the steady state is regulated independently of the mechanisms that restrict the initial proliferative burst of T cells. Such a bifurcation essentially restricts application of the TAT model to the time frame of the post-expansion adaptive tolerance phase in our system.

Two other complexities that are challenging for the TAT model are that PCC-lo–adapted T cells display nearly twice as much CD69 and 1.4-fold higher polymerized actin in vivo compared with the PCC-hi–adapted cells. These observations suggest that in the post-adaptive scenario greater amounts of TCR signals are being transduced by the PCC-lo–adapted T cells in response to the ambient PCC-lo stimulus itself, relative to transduction by the PCC-hi–adapted T cells of the PCC-hi stimulus in vivo. Such an over (in PCC-hi) and/or under (in PCC-lo) compensation is not predicted by a model that envisions direct balancing of antigen presentation strength by negative feedback operating on TCR proximal signaling events. Adaptation therefore seems to be primarily concerned with restricting the proliferative potential of T cells after an initial wave of response in the context of persistence of stimulus. Because multiple pathways downstream of the TCR contribute differentially to entry into cell cycle, different extents of negative feedback may need to be initiated in different pathways to effectively control the cell cycle. Persistent signaling could continue to recruit more inhibition into the active transduction pathways of the anergic T cells, until the integrated nuclear signal is only sufficient to allow a basal turnover, which is interestingly also observed for memory T cells in vivo (29). In the case of the weaker stimulus environment there would be less inhibition required to reduce the turnover, leading to less complete inhibition of some of the signaling pathways. This could allow for TCR signaling to continue triggering consequences that are elicited at a lower threshold of signaling in those pathways alone. For example, an incomplete block of the MAPK pathway or of the JNK pathway might allow for the expression of CD69 or apoptotic processes, respectively, in the PCC-lo environment, even after turnover has been constrained. This could explain the slow but significant loss of T cells (>50%) in the PCC-lo host after 3–4 mo. Furthermore, because the expression of negative feedback is also likely to depend on the strength of the signal (30), T cells in the lower stimulus environment might also take longer to recruit adequate feedback to reach the adaptive state. This is consistent with the delay for entry into tolerance observed in the PCC-lo host as measured by the kinetics of IL-2 and IFN-γ down-regulation.

A critical deficiency in the literature pertaining to the tunability of T cell activation thresholds is the inability of many experimental systems to distinguish selection for existing variability in the population from tuning of an individual cell's threshold. This is largely because of the use of polyclonal populations or TCR transgenic cells with endogenously rearranged receptors, which impart variable properties to the starting population that can then be selected upon. In contrast, our original T cell cohort is derived from a RAG2-null TCR transgenic mouse in which >95% of cells are $CD69^-$, $CD25^-$, and $CD44^{lo}$ and express similar levels of TCR. Commensurate with this homogeneity, the cells also behave as a fairly synchronous population during in vivo proliferation in the PCC-hi host (initial divisions follow a Gaussian distribution with a SD < 1). Furthermore, after adaptation has been established, phalloidin staining shows a homogeneous population of differentially responsive T cells in the two environments. Therefore, our observations are most likely documenting the adaptive changes acquired at the level of individual cells from a homogeneous pot of naive T cells.

Nonetheless, as a theoretical alternative to the TAT model to explain our results, it is still possible to envision complex processes of selection operating on subtle intrinsic differences in the monoclonal naive T cell population stimulated at less than optimal conditions. A selection hypothesis that assumes heritable differences in our starting population of TCR transgenic T cells is unlikely to explain our observations because the hyporesponsiveness is reversible in the absence of antigen (18). A more complex model of selection, however, could assume that individual T cells constitutively oscillate in their responsiveness over an indeterminate range. Such a model would explain our results by arguing that cells that are sufficiently responsive at any given instant to be activated by the ambient stimulus would be depleted by deletion or migration. The lymphoid compartment would thus only maintain cohorts of T cells that display responsiveness oscillations lower than that stimulatable by the presented antigen. Hence, two different ambient stimuli would produce two populations with different mean responsiveness. Such an "oscillating responsiveness" scenario shares an attractive feature of the TAT model in that it predicts T cell responsiveness to be a dynamic and variable property rather than a binary gate. In contrast to the TAT model, however, it does not subsequently ascribe tunability to the intrinsic TCR thresholds. Although the two models invoke different mechanisms (selection vs. tuning), the endpoint in either case is the same, i.e., the availability of a dynamic range of T cell responsiveness that can effectively be adapted to a given persistent stimulus.

Because the oscillating responsiveness model does not allow for cell-intrinsic tuning, it would predict that T cell responses should be proportional to the ambient stimulus, i.e., cells in the PCC-hi environment should always make equal or greater responses than those in the PCC-lo environment. Our observations of both a lower MFI of CD69 and a lower level of actin polymerization in the PCC-hi–adapted T cells is contrary to such a prediction. This discrepancy is thus a strong argument for the existence of cell-intrinsic tunability as envisioned by the TAT model. Still, the complexity of the cell-intrinsic adaptive behavior that we have observed calls for further refinement of the theoretical models to better define the molecular networks driving TATs in peripheral $CD4^+$ T cells.

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