Tolerant CD8 T Cells Induced by Multiple Injections of Peptide Antigen Show Impaired TCR Signaling and Altered Proliferative Responses In Vitro and In Vivo

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The mechanisms responsible for peripheral CD8 T cell tolerance to foreign Ags remain poorly understood. In this study we have characterized the state of CD8 T cell tolerance induced in F5 TCR transgenic mice by multiple peptide injections in vivo. The tolerant state of CD8 T cells is characterized by impaired proliferative responses, increased sensitivity to cell death, and failure to acquire cytotoxic effector function after in vitro antigenic challenge. In vivo monitoring of CD8 T cell proliferation using 5-carboxyfluorescein diacetate succinimidyl ester showed that a large subset of the tolerant T cell population failed to divide in response to peptide. TCR down-regulation could not account for this loss of responsiveness to Ag since recombination-activating gene-1 (RAG-1)−/− F5 CD8 T cell responses were similar to those of RAG-1−/−×F5 × RAG-1−/− F1 T lymphocytes, which express lower levels of the transgenic TCR. Analysis of early signal transduction in tolerant CD8 T cells revealed high basal levels of cytoplasmic calcium as well as impaired calcium mobilization and tyrosine phosphorylation after cross-linking of CD3ε and CD8α. Together these data indicate that repeated exposure to soluble antigenic peptide in vivo can induce a state of functional tolerance characterized by defective TCR signaling, impaired proliferation, and increased sensitivity to cell death. The Journal of Immunology, 1998, 161: 5260–5267.

T cell tolerance is achieved through a combination of thymic and peripheral events that render the peripheral T cell pool unresponsive to self Ags. Thymocytes reactive for self peptides are either deleted (1–3) or rendered functionally unresponsive (4, 5). However, because particular self peptides are not presented in the thymus, some autoreactive T cells reach the periphery in normal individuals. A number of mechanisms prevent these self-reactive T lymphocytes from initiating autoimmune responses. They include immune ignorance of Ags expressed at low levels or in sequestered sites (6–9), anergy (10–14), suppression (15–18), and deletion (19–22). Upon exposure to cross-reactive Ags, autoreactive T cells that migrate to the periphery may be activated and trigger autoimmune reactions (8).

The incidence of autoimmune diseases and the development of organ transplants have highlighted the need for protocols aimed at inducing a state of peripheral T cell tolerance. This can be achieved following in vivo treatment with Abs directed against TCR/CD3 complex components (23–26), Abs to CD4 or CD8 co-receptor molecules (27), or superantigens (28–31). However, these protocols lead to partial or total immunosuppression. The induction of tolerance to a given Ag has therefore been investigated as a means of blocking specific responses without affecting the entire peripheral T cell pool. Peripheral tolerance to foreign antigenic peptides recognized by CD4 T cells can be established after exposure to soluble Ags administered orally (15, 16) or systemically (32–35). While oral administration of Ag leads to TGF-β-mediated immunosuppression (15, 16), systemic exposure to Ag is thought to load APCs without promoting their maturation and expression of costimulatory molecules. Deletion often accounts for the lack of specific T cell responses after treatment with polyclonal activators or antigenic peptides in vivo (30, 33), but survival of CD4 T cells that have become unresponsive to Ag in vivo has also been described (35).

Studies performed on CD4 T lymphocytes in vitro have indicated that induction of unresponsiveness or anergy leads to the inability of these cells to produce IL-2 and/or proliferate upon restimulation (36–38). Anergy induction also leads to alterations in the early signal transduction events that follow Ag receptor engagement. These include altered regulation of the inositol phosphates and calcium metabolism (39, 40), constitutive Fyn kinase activity (41), a partial block in TCR/CD3-induced tyrosine phosphorylation (39, 40), and diminished activation of the signaling pathways located downstream of ras (42, 43).

For CD8 T cells, multiple systemic exposures or high doses of Ag induce tolerance in vivo (12, 44–46). However, the mechanisms responsible for tolerance of specific CD8 T cells remain poorly understood. Deletion of Ag-specific T cells (12, 20) and down-regulation of TCR surface levels (14) have both been observed in TCR transgenic systems after tolerance induction with exogenous Ags. There is also evidence indicating that a subset of the CD8 T cells is rendered anergic after tolerance induction. These cells can survive in the absence of Ag and recover responsiveness over time (47).
We have previously shown that a single injection of soluble antigenic peptide induces a population of long-lived CD8 T cells that is hyper-reactive to Ag in vitro (48). In the present study we have used the F5 TCR transgenic system (49) to characterize the CD8 T cells that survive a tolerance induction protocol consisting of multiple exposures to soluble antigenic peptide. Our data show that 7 days after the last of three peptide injections administered at 4-day intervals, surviving CD8 T cells proliferate poorly in vitro and in vivo and have lost the ability to differentiate into cytotoxic effectors after in vivo stimulation in the absence of exogenously added cytokines. In addition, the tolerant F5 CD8 T cells are highly sensitive to cell death in vitro. Analysis of early signal transduction pathways revealed that these cells also had constitutively elevated cytoplasmic calcium levels and impaired early signal transduction in response to co-cross-linking of CD3 with CD8.

Materials and Methods

Mice and immunizations

C57BL/10, F5, RAG-1−/− F53 TCR transgenic and RAG-1−/− mice were gifts from D. Kioussis (49). All animals were bred in the institute’s animal facility. Immunizations were performed by injecting 50 μl of the A/NT/60/68 influenza virus nucleoprotein peptide Ala-Ser-Asn-Glu-Asn-Met-Asp-Ala-Met (NP, NeoSs Laboratories, Strasbourg, France) in PBS into the peritoneal cavity. Control mice either were not treated or were injected with PBS alone.

Cell culture and proliferation assays

Spleen and lymph node T cells were cultured in RPMI 1629 (Life Technologies, Gaithersburg, MD) supplemented with 2 mM l-glutamine (Life Technologies), 10 μg/ml gentamicin (Life Technologies), 6% FCS (Boehringer Mannheim, Mannheim, Germany), and 5 × 10−5 M β-ΜΕ. F5 transgenic T cells (5 × 105 cells/well) were activated in 96-well plates with graded doses of peptide and 2 × 103 irradiated (3000 rad) C57BL/10 spleen cells and in the presence or the absence of 5% cell culture supernatant containing IL-2. The final IL-2 content ranged from 100–150 U/ml. After two more washes with PBS/horse serum/NaN₃, cells were incubated for 5 min at 37°C with homemade biotinylated mAbs to CD3 (clone, CD8 (YTS 191.1, Caltag) and B220 (Sigma, St. Louis, MO). Analysis was performed on a Becton Dickinson FACS-Star Plus. The UV excitation (351–364 nm) was provided by an argon laser; blue emission was detected at 480–520 nm, and violet emission was detected at 383–407 nm. Cells that were negative for PE staining were gated on CD8+CD4−. For activation, samples of 2 × 105 LN cells were incubated for 5 min at 37°C with homemade biotinylated mAbs to CD3ε (mAb 145 2C11) and CD8α (YTS169.4).

Avidin was added 2 min after the beginning of the assay. Results are expressed as the mean indo ratio over time in the CD4−B220+ population.

Tyrosine phosphorylation assays

CD8 lymph node T cells were purified with magnetic beads using a negative selection strategy. Briefly, total lymph node cells were incubated with anti-CD4 (GK1.5) and anti-B220 (RA3 6B2) hybridoma culture supernatants for 45 min at 4°C. Cells were then washed three times and incubated for 30 min at 4°C with magnetic beads coupled to goat anti-rat IgG (H & L) (Biomag, Perseptive Diagnostics, U.K.) at a ratio of 15 beads/cell. As determined by flow cytometric analysis, CD8 T lymphocytes represented 90–95% of the cells that remained after magnetic depletion. Samples of 1 × 10⁶ CD8 T cells were incubated at room temperature with biotinylated mAbs to CD3ε (mAb 145 2C11) and CD8α (YTS169.4) for 5 min. After a 3-min incubation with avidin at 37°C, cells were rapidly pelleted and lysed for 15 min in 250 μl of ice-cold 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% aprotinin, and 1 mM PMSF buffer containing 2 mM sodium vanadate. Lysates were cleared at 14,000 rpm for 10 min. Proteins were then separated by electrophoresis on 4–20% SDS-acrylamide gradient gels (Novex, San Diego, CA) and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Immunoblots were saturated for 1 h with 6% BSA in Tris-buffered saline and incubated for 18 h with 0.75 μg/ml of a rabbit anti-phosphotyrosine antiserum (Zymed, San Francisco, CA) in 6% BSA in TBS. Detection was performed using pig anti-rabbit peroxidase Abs (Dako, Copenhagen, Denmark) followed by enhanced chemiluminescence.

Results

CD8+ T cells from F5 TCR transgenic mice treated with multiple peptide injections in vivo are tolerant in vitro

Multiple systemic exposures to a soluble viral antigenic peptide has been shown to induce tolerance of CD8 peripheral T cells (44–46, 52). We first sought to determine how this tolerance induction protocol affected the peripheral CD8 T cell pool in F5 TCR transgenic mice. F5 mice were given three injections of the cognate influenza virus NP366–374 peptide at 4-day intervals. Seven days after the last peptide injection, the number of F5 CD8 T cells that could be recovered in the lymph nodes represented at least 30% of that in control animals. A subtle decrease in the expression levels of the transgenic TCR Vβ11 chain, CD3ε, and CD8α could be detected on the surface of CD8 T cells stimulated in vivo (Fig. 1A). A survey of surface molecules known to be up-regulated after in vivo T cell stimulation revealed that CD44 and Ly-6C were the only markers that were up-regulated on the surface of CD8 T cells stimulated 7 days before analysis with either single or multiple

Labeling of cells with CFSE

The method used for labeling cells with CFSE is a modification of the protocol described by Weston and Parish (50). Briefly, T cells were resuspended at a concentration of 1–5 × 10⁷/ml in fully supplemented growth medium before the addition of CFSE at a final concentration of 10 nM. Cells were then incubated for 10 min at 37°C, and the reaction was stopped by adding 5 vol of ice-cold medium. After two washes, CFSE-labeled cells were resuspended in growth medium for in vitro culture or in PBS for i.p. injection into C57BL/10 recipients. To achieve higher levels of CFSE fluorescence, RAG-1−/− F5 and F1 (RAG-1−/− F5 × RAG-1−/−) T cells were labeled with CFSE for 13 min instead of 10 min for some in vivo assays.

Analysis of intracellular calcium mobilization

This method has been described in detail previously (51). Total lymph node cells were loaded at a final concentration of 10⁷/ml in fully supplemented RPMI 1629 containing the acetomethylster of indo-1 at a concentration of 2 mM and 0.2% FI27 (Molecular Probes, Junction City, OR). Loading was conducted at 37°C for 40 min in the dark. Cells were then washed, resuspended in fully supplemented medium, and stained at 4°C with PE-coupled Abs directed against CD4 (YTS 191.1, Caltag) and B220 (Sigma, St. Louis, MO). Analysis was performed on a Becton Dickinson FACS-Star Plus. The UV excitation (351–364 nm) was provided by an argon laser; blue emission was detected at 480–520 nm, and violet emission was detected at 383–407 nm. Cells that were negative for PE staining were gated on CD8+CD4−. For activation, samples of 2 × 10⁶ LN cells were incubated for 5 min at 37°C with homemade biotinylated mAbs to CD3ε (mAb 145 2C11) and CD8α (YTS169.4). Avidin was added 2 min after the beginning of the assay. Results are expressed as the mean indo ratio over time in the CD4−B220+ population.

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peptide injections (data not shown). When cells stimulated multiple times in vivo were cultured in vitro in the presence of irradiated syngenic spleen cells and graded doses of Ag, they proliferated poorly compared with control populations (Fig. 1B) or CD8 T cells primed by a single peptide injection (data not shown). Similar results were obtained when proliferation was measured at different times after in vitro stimulation (data not shown). Proliferative responses were partially restored if tolerant F5 CD8 T cells were stimulated in the presence of exogenously added IL-2 (Fig. 1B).

These results indicated that multiple peptide injections in vivo rendered F5 CD8 T cells hyporesponsive to peptide stimulation in vitro.

Loss of ex vivo cytotoxic effector function has also been reported in tolerant virus-specific CD8 T cells (45). To determine whether F5 tolerant T cells were capable of becoming CTL effectors, control and tolerant F5 CD8 T lymphocytes were activated in vitro for 3 days and tested for the ability to lyse peptide-loaded P815-D8 targets. The data presented in Fig. 2 show that tolerant T cells failed to become efficient CTL. However, in the presence of IL-2, tolerant T cells were able to differentiate into cytotoxic effector cells. Based on the hyporesponsive phenotype described above, F5 CD8 T cells stimulated by multiple peptide injections in vivo will be called tolerant.

Altered proliferation and increased cell death among tolerant F5 CD8 T cells in vitro

A number of mechanisms could account for the poor peptide-specific proliferative responses of tolerant T cells observed in vitro. These include a lower frequency of peptide-responsive cells, an increased rate of cell death, and slow or lack of proliferation in response to Ag in vitro. To follow the fate of tolerant CD8 T cells in vitro, we have used the fluorescent dye CFSE, which allows tracking of cell divisions using flow cytometry. Divisions could be detected as early as 40 h of in vitro stimulation in both control and tolerant CD8 T cell populations and at peptide doses ranging from 0.15–5 nM (data not shown). After 3 days in culture in the presence of 5 and 1.5 nM peptide, several divisions could be detected
in both control and tolerant F5 CD8 T cell populations (Fig. 3A). However, the number of cell divisions accomplished by activated tolerant T cell populations after 3 days in culture was lower than that observed in control F5 CD8 T cells. Moreover, at this time point, the number of viable tolerant CD8 T cells was significantly lower than that in control samples. At low Ag doses (0.5 and 0.15 nM) and after 3 days in culture, these numbers were consistently 10–20 times lower in tolerant T cell populations than in control samples. By comparison, F5 CD8 T cells stimulated by a single peptide injection 7 days before analysis performed the same number of divisions as naive cells (Fig. 3B). Together, these data show that when tolerant F5 CD8 T cells were cultured in vitro they performed fewer divisions in response to peptide and were more sensitive to cell death compared with naive populations.

Ag-specific proliferative responses of tolerant F5 CD8 T cells are impaired in vivo

To determine whether hyporesponsiveness of tolerant CD8 T cells was also detectable in vivo, control and tolerant CD8 T lymphocytes were labeled with the fluorescent dye CFSE and injected into C57BL/10 recipients. Three days later, recipient mice were injected with antigenic peptide, and CD8 T cell divisions were monitored 72 h later. In these assays CD8 T cells from both F5 and RAG-1−/− F5 mice were used. As shown in the following section, multiple peptide injections into RAG-1−/− F5 mice also induced a state of in vitro hyporesponsiveness (see Fig. 5). The data presented in Fig. 4 show that while the overall majority of naive RAG-1−/− F5 CD8 T cells recovered from the lymph nodes of recipient mice were capable of dividing in response to antigenic challenge (Fig. 4A), only a minor fraction of the tolerant T cells appeared to have done so (Fig. 4B). As a further indication of the diminished proliferation of tolerant CD8 T cells, we found that there was no increase in the percentage of CFSE-labeled lymph node CD8 T cells following peptide injection. In contrast, control CFSE-labeled CD8 T cell populations increased three- to sevenfold under the same experimental conditions (Fig. 4, A and B, and data not shown). This was not dependent on the RAG-1−/− background, since similar observations were made when tolerant F5 CD8 T cells were injected into C57BL/10 mice (data not shown).

These results provide direct in vivo evidence that a significant fraction of the tolerant CD8 T cell population was not capable of dividing in response to a dose of Ag that triggers the large majority of control transgenic CD8 T cells to divide. They also argue that peptide tolerance is not simply the result of a selection of cells that did not respond to Ag, since the overall majority of naive RAG-1−/− F5 CD8 T cells divided in response to peptide challenge in vivo.

Role of cell surface transgenic TCR expression levels in tolerant CD8 T cell response

The data presented above indicate that multiple injections of an agonist peptide in vivo lead to hyporesponsiveness in vitro and in vivo. Expression of a second TCR α-chain has been described in mouse T cells (53, 54). Since a clonotypic Ab is not available in the F5 system, we could not rule out the possibility that the F5 CD8 T cells that survived this tolerance induction protocol were, in fact, cells that did not fully respond to the antigenic peptide. It was thus conceivable that multiple peptide injections selected cells that expressed lower levels of the transgenic F5 TCR α-chain. F5 mice deficient for the RAG-1 enzyme were therefore used to determine whether such cells contributed to the unresponsiveness to peptide challenge in vitro. In these mice, only the transgenic TCR is expressed on the T cell surface. RAG-1−/− F5 CD8 T cells that survived multiple peptide stimulations in vivo expressed lower levels of Vβ11 on the cell surface (Fig. 5A). This down-regulation of Vβ11 expression was more pronounced than that seen in the F5 CD8 T cells and corresponded to a twofold decrease (see Fig. 1). As observed in F5 peripheral CD8 T cells, a small but reproducible decrease in CD8α surface expression was also detected in RAG-1−/− F5 T cells stimulated by multiple peptide injections in vivo.

FIGURE 4. Tolerant CD8 T cells are hyporesponsive to peptide in vivo. Naive (A) and tolerant (B) RAG-1−/− F5 CD8 T cells were labeled with CFSE and adoptively transferred by i.p. injection into C57BL/10 recipients. Three days later, mice were challenged with PBS (thin lines) or with 50 nmol of peptide (thick lines). Lymph node T cells were harvested on day 6 post-transplant and were stained with anti-CD8 coupled to PE. Results show CFSE fluorescence in a viable cell population gated for CD8 expression. These results are representative of three independent experiments.

FIGURE 5. Surface phenotype and proliferative responses of RAG-1−/− F5 and F1 (RAG-1−/− F5 × RAG-1−/−) CD8 T cells. A, Spleen CD8 T cells from naive RAG-1−/− F5, peptide-treated RAG-1−/− F5, or naive F1 mice were tested for surface expression of CD8 and Vβ11 7 days after the last of three peptide injections. Results from one representative experiment of three are presented. B, In vitro proliferative responses of naive RAG-1−/− F5, peptide-treated RAG-1−/− F5, and naive F1, CD8 T cells. CD8 T cells (5 × 10⁶) were stimulated for 3 days with graded doses of peptide in the presence or the absence of IL-2 as described in Materials and Methods. C, Proliferation of naive RAG-1−/− F5 and RAG-1−/− F5 × RAG-1−/− F1 CFSE-labeled CD8 T cells in vivo was measured as described in Fig. 4.
were similar to those of control RAG-1 knockout mice. CD8 T cells in vitro, we investigated whether hyporesponsiveness correlated with Ag receptor-mediated early TCR signal transduction. They include increased baseline activity of Fyn (42), inhibition of Ag receptor-mediated early tyrosine phosphorylation events (26, 40, 41), and diminished intracellular calcium mobilization (40, 41). As multiple injections of antigenic peptide into F5 CD8 T cells lead to inhibition of F5 CD8 T cell proliferation in vivo (Fig. 5A). However, despite lower cell surface levels of TCR, F1 (RAG-1−/− F5×RAG-1−/−) CD8 T cell responses to peptide were similar to those of control RAG-1−/− F5 CD8 T cells in vitro as measured using thymidine incorporation (Fig. 5B). Similar observations were made when comparing RAG-1−/− F5 and F1 (RAG-1−/− F5×RAG-1−/−) CD8 T cell responses to peptide using CFSE-labeled cells transferred into C57BL/10 recipients (Fig. 5C).

These results argue that lower levels of cell surface transgenic TCR cannot alone account for impaired proliferative responses in the CD8 T cell population that has been stimulated by multiple peptide injections in vivo.

Multiple exposures to antigenic peptide in vivo lead to a partial block of TCR signal transduction

T cell activation using partial agonist peptides (55), superantigens (56, 57), and anti-CD3 Abs (26, 39) in vitro or in vivo induces a state of T cell unresponsiveness that has been linked to alterations in TCR signal transduction. They include increased baseline activity of Fyn (42), inhibition of Ag receptor-mediated early tyrosine phosphorylation events (26, 40, 41), and diminished intracellular calcium mobilization (40, 41). As multiple injections of peptide in vivo lead to inhibition of F5 CD8 T cell proliferation in vitro, we investigated whether hyporesponsiveness correlated with early TCR signaling defects. CD8+ T cells from F5 mice injected three times with antigenic peptide were tested for their ability to respond to co-cross-linking of CD3 with CD8. In these experiments, F5 CD8 T cells were used because down-regulation of CD3ε was less pronounced than that seen in the RAG-1−/− background. In tolerant T cells, basal levels of intracellular free calcium were higher than those in control populations (Fig. 6A). In addition, intracellular free calcium responses (Fig. 6B) and tyrosine phosphorylation of several cellular substrates (Fig. 7) were both significantly reduced in tolerant CD8 T cells.

These results indicate that following multiple peptide injections, F5 CD8 T cells are defective for signaling through the TCR complex.

Discussion

The results presented in this study show that multiple systemic administrations of antigenic peptide into F5 TCR transgenic mice lead to functional defects in peptide-specific CD8 T cells. These cells displayed diminished proliferative responses to peptide both in vitro and in vivo. They also failed to differentiate into cytotoxic effectors when restimulated in vitro with peptide in the absence of exogenously added IL-2. Ag receptor down-regulation did not account for tolerant responses in vitro and in vivo. Instead, analysis of early signal transduction events revealed constitutively elevated resting levels of intracellular free calcium as well as impaired signaling after TCR/CD3 cross-linking with CD8.

The hyporesponsive phenotype described in this study does not appear to be linked to activation-induced T cell short term desensitization. Indeed, the proliferative responses of CD8 T cells activated in vivo by a single peptide injection 7 days before analysis were identical with those of naive populations. This is in agreement with the observation that a single exposure to peptide administered 10 days before exposure to virus may protect mice from infection with lymphocytic choriomeningitis virus (44). It is also consistent with induction of a memory-like F5 CD8 T cell population after a single exposure to antigenic peptide in vivo (48). In addition, we have found that 7 days after a single peptide injection, calcium mobilization triggered by co-cross-linking of CD3 and CD8α...
CD8 was comparable to that seen in naive populations (data not shown).

A number of mechanisms have been proposed to account for hyporesponsiveness of tolerant peripheral T cells. One of these argues that T cell hyporesponsiveness after tolerance induction results from selection of a pre-existing population that cannot respond to peptide. Our results strongly argue against such a model by providing evidence indicating that hyporesponsiveness affected a population of cells that had responded to the first encounter with peptide. Indeed, using naive CFSE-labeled RAG-1−/− F5 CD8 T cells transferred into C57BL/10 recipients, we have shown that the overall majority of RAG-1−/−/F5 CD8 T cells had proliferated 3 days after peptide injection. These data are in agreement with our previous report showing that the bulk of F5 CD8 T cells incorporate bromodeoxyuridine in response to a single peptide injection (48) and make it unlikely that surviving CD8 T cells are derived from a RAG-1−/− F5 CD8 T cells subset that did not respond to Ag.

Another mechanism that could account for hyporesponsiveness in the surviving Ag-specific T cell population involves a decrease in the number of cell surface TCR molecules capable of recognizing the peptide. In the F5 system, this could be the consequence of a second TCR α-chain rearrangement that has been reported in humans (58) as well as in normal and TCR transgenic mice (53, 54). One consequence of dual TCR α-chain rearrangement is the expression of two Ag receptors. As a result, the number of cell surface TCRαβ receptors capable of recognizing a given Ag may be decreased. Since the ability to transduce activation signals correlates with TCR surface levels (59) in T cell clones, responses to limiting doses of Ag may be diminished in T cells expressing two Ag receptors. In vivo evidence for such a mechanism comes from studies in transgenic systems in which skin grafts are not rejected by CD8 T cells expressing lower levels of the Ag receptor (14, 60). In the F5 model, cells expressing a second TCR would be selected after multiple stimulations in vivo and would become the bulk of the peripheral CD8 T cell population that survived tolerance induction. The fact that RAG-1−/−/F5 CD8 T cells that cannot express endogenous TCR α-chain could be rendered tolerant argues against such a mechanism.

Following multiple peptide injections, CD8 T cells from RAG-1−/−/F5 mice show some down-regulation of the TCR. In some systems, this is thought to be responsible for the lack of responsiveness of tolerant T cell populations to Ag (14, 60). However, using T cells that express cell surface levels of TCR Vβ11 comparable to those detected on the surface of tolerant RAG-1−/−/F5 CD8 T lymphocytes (see Fig. 5), we found that this had no influence on the proliferative responses of naive cells to peptide both in vitro and in vivo. These results show that mechanisms other than TCR down-regulation are responsible for the tolerant phenotype of F5 CD8 T cells. Indeed, modifications of TCR signaling have been demonstrated in anergic CD4 T cells in vitro (39, 40).

In support of such a model, we found that F5 CD8-tolerant T cells displayed fundamental alterations in early TCR signaling pathways. We first detected high levels of intracellular Ca2+, an observation that has been reported in anergic CD4 T cell clones (41). We further demonstrated that tolerant CD8 T cells were partially defective for intracellular signals such as cytoplasmic free calcium increases and tyrosine phosphorylation triggered by TCR/CD3 co-cross-linking with CD8. These results are consistent with previous reports showing that T cells that survive in vivo stimulation with superantigens display altered Ag receptor signaling (56, 57). They also suggest that alterations in intracellular calcium metabolism and TCR signaling contribute to block tolerant CD8 T cell responses in vitro and in vivo. Further analysis will be required to determine whether tolerant CD8 T cells share with anergic CD4 T cell clones increased Fyn activity (41), high Rap-1-GTP content (61), and impaired TCR-mediated activation of ERK and JNK (42, 43).

Using CFSE-labeled cells adoptively transferred into C57BL/10 recipients, we have shown that while the overall majority of naive F5 CD8 T cells did divide in response to peptide, a large fraction of the tolerant T cell population did not proliferate in the recipient’s lymph nodes. These results clearly indicate that 10 days after the last exposure to peptide and 3 days after transfer into an environment free of Ag, a significant proportion of the F5 TCR transgenic CD8 T cells remained unresponsive to peptide in vivo. To determine whether cytokines produced by naive F5 CD8 T cells could drive tolerant T cells to divide in vivo, we also injected tolerant CFSE-labeled F5 CD8 T cells into F5 recipients. In these mice, the large majority of peripheral CD8 T cells are activated and divide in response to the same dose of peptide (39). However, we found no evidence suggesting that tolerance could be reversed under these experimental conditions (data not shown).

Some tolerant CFSE-labeled CD8 T cells were capable of dividing in response to peptide stimulation in C57BL/10 recipients. These cells did not correspond to a subset of CD8 T cells that has escaped tolerance induction in the donor. Indeed, the division profiles that can be detected 3 days after peptide injection in the recipient mice were strikingly different when comparing naive and tolerant F5 CD8 T cells. Activation of naive cells leads to a normal distribution of division peaks, with a maximum at four divisions. By contrast, division peak distribution was not normal when tolerant T cells were analyzed, since the peak corresponding to one division contained the largest number of cells. This observation could mean that tolerant CD8 T cells that divided also migrated rapidly out of the lymph nodes. However, based on our in vitro results showing that CFSE-labeled tolerant T cells were highly sensitive to cell death, it seems more likely that those F5 tolerant T cells that responded to peptide stimulation in vivo died.

By comparing the percentages of control and tolerant CFSE-labeled F5 CD8 T cells detected in the lymph nodes of C57BL/10 recipients challenged with PBS, we also found that they were consistently lower in mice injected with tolerant F5 CD8 T cells (data not shown). One possibility is that the homing of naive and that of tolerant CD8 T cells are different. However, there is also evidence suggesting that tolerant T cells may have a shorter life span in vivo. First, T cells rendered anergic in vivo gradually disappear when parked in a recipient that does not express the Ag (62). Second, recent reports have shown that survival of naive TCRαβ T cells in the periphery is dependent on an environment expressing the restricting MHC (63–65). These findings show that a survival signal relayed by TCR is required for maintenance of mature T cells in peripheral lymphoid organs. This is compatible with a model proposed by Raff and co-workers (66) in which mammalian cells constitutively express proteins involved in triggering apoptosis, such that survival of these cells would require signals to inhibit this default death program. Since tolerant F5 T cells are partially defective for TCR and coreceptor signaling in our system, it is conceivable that the amount of signal transduced by the Ag receptor complex and/or CD8 upon interaction with H-2Dk is not sufficient to ensure survival of these cells in vitro and in vivo.

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