Anergy in Peripheral Memory CD4^{+} T Cells Induced by Low Avidity Engagement of T Cell Receptor

Saied Mirshahidi,[1] Ching-Tai Huang,[2]
and Scheherazade Sadegh-Nasseri[1]

[1]Department of Pathology and [2]Department of Oncology, Johns Hopkins University, School of Medicine, Baltimore, MD 21205

Abstract

Induction of tolerance in self-reactive memory T cells is an important process in the prevention of autoimmune responses against peripheral self-antigens in autoimmune diseases. Although naive T cells can readily be tolerized, memory T cells are less susceptible to tolerance induction. Recently, we demonstrated that low avidity engagement of T cell receptor (TCR) by low densities of agonist peptides induced anergy in T cell clones. Since memory T cells are more responsive to lower antigenic stimulation, we hypothesized that a low avidity TCR engagement may induce tolerance in memory T cells. We have explored two antigenic systems in two transgenic mouse models, and have tracked specific T cells that are primed and show memory phenotype. We demonstrate that memory CD4^{+} T cells can be rendered anergic by presentation of low densities of agonist peptide–major histocompatibility complex complexes in vivo. We rule out other commonly accepted mechanisms for induction of T cell tolerance in vivo, such as deletion, ignorance, or immunosuppression. Anergy is the most likely mechanism because addition of interleukin 2–reversed anergy in specific T cells. Moreover, cytotoxic T lymphocyte antigen (CTLA)-4 plays a critical role in the induction of anergy because we observed that there was increased surface expression of CTLA-4 on anergized T cells, and that injection of anti–CTLA-4 blocking antibody restored anergy in vivo.

Key words: T cell tolerance • autoimmunity • transgenic mice • CTLA-4 • antigen presentation

Introduction

Induction of tolerance in self-reactive memory T cells is essential for prevention of autoimmune responses against peripheral self-antigens in autoimmune diseases. However, although naive T cells can be tolerized successfully (1, 2), memory T cells are less susceptible to tolerance induction in vivo (3). Differences that distinguish naive from memory T cells may contribute to the differential susceptibility to tolerogenic signals. For example, naive and memory CD4^{+} T cells express different patterns of adhesion molecules, such as CD44, intercellular adhesion molecule 1, LFA-1, and CD62L, or signaling molecules such as CD45. Because of an increased expression of adhesion receptors, memory T cells require less costimulation than do naive cells, and are able to respond faster and to lower densities of antigenic challenge (3). Some differences at the levels of TCR-induced signal-

ing pathways may also contribute to a lower activation threshold for memory cells. Specifically, stimulation through TCR/CD3 in murine naive versus memory CD4^{+} T cells leads to differential phosphorylation of proteins involved in signal transduction (4). These characteristics may enable memory T cells to respond positively to stimulation by all APCs, including resting B cells (5). In agreement with these findings it has been documented that lack of costimulatory signals does not lead to tolerance in memory T cells (2).

The role of CTLA-4 in maintaining peripheral tolerance is well established. Recently, Greenwald et al. (1) clearly demonstrated an essential role for CTLA-4 in regulating the induction of anergy in vivo. It is reported that CTLA-4 is produced and stored in endosomal vesicles in activated/memory, but not naive T cells (6). Thus, when needed, CTLA-4 might readily be expressed on the cell surface and act to regulate overstimulation of activated/memory T cells.

Recently, we have demonstrated that low avidity engagement of T cell receptor by low densities of agonist peptides induced anergy in T cell clones (7). Thus, we hypothesized that low avidity TCR engagement may also

drive memory T cells to a state of anergy in vivo. To test this hypothesis, we designed two experimental systems using two transgenic (Tg)[*] mouse models. In one, monoclonal 6.5 TCR Tg mice, specific for hemagglutinin (HA)$_{110-120}$, and in the other, HLA-DR1 Tg mice, with a full repertoire of T cells, were tested. We observed that presentation of low densities of agonist peptides in complex with MHC class II induced tolerance in specific memory CD4$^+$ T cells. We demonstrated that tolerance was not due to ignorance or active suppression/immunoregulation. Furthermore, by studying specific T cells tracked by specific mAb or peptide–MHC II oligomers, we established that tolerance was due to anergy but not deletion. Moreover, induction of anergy required signaling through CTLA-4.

Materials and Methods

Mice. TCR Tg mice line 6.5 that expresses an α/β T cell receptor recognizing an I-E^d–restricted HA$_{110-120}$ on a B10 background was used. NonTg B10.D2 mice at 6–8 wk of age were purchased from The Jackson Laboratory. HLA-DR1 (DR B1*0101) Tg mice (Merck) at 8–10 wk of age were studied. The chimeric HLA-DR1 molecule comprised a peptide-binding groove derived from the human DR1 sequence and a CD4-binding domain from I-E^f mice. The mice were housed in The Johns Hopkins University animal facilities under virus-free conditions. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University, School of Medicine.

Peptides. The peptides, influenza virus HA$_{306-318}$ (PKYV-KQNTLKLAT) and HA$_{109-120}$ (SSFERFEIFPKE), were synthesized by Peptide Express. The peptides were >90% pure as analyzed by reverse-phase HPLC.

Immunizations. To determine the immunogenicity of the HA$_{306-318}$, HLA-DR1 Tg mice were immunized subcutaneously at the base of the tail with 10 nmol of HA$_{306-318}$ emulsified at a 1:1 (vol/vol) in Complete Freund's Adjuvant (CFA; Sigma-Aldrich). After 2, 3, 5, 7, or 12 wk, mice were injected subcutaneously with increasing concentrations (0–50 nmol) of HA$_{306-318}$ in Incomplete Freund's Adjuvant (IFA; Sigma-Aldrich). 9 d after the second injection, inguinal lymph nodes were removed and cells were used for assays.

Adoptive Transfer. CD41 cells (2.5 × 10^6), positive for the clonotypic TCR, prepared from pooled lymph nodes and spleens of TCR Tg donors, were resuspended in 200 μl of sterile HBSS (Life Technologies) and injected through the tail vein of B10.D2 recipient mice. 3 d after transfer, mice were immunized (subcutaneously) with 15 nmol of HA$_{109-120}$ emulsified at a 1:1 (vol/vol) ratio with CFA. After 5 wk, recipient mice were injected with increasing concentrations of HA$_{109-120}$ (0–10 nmol) mixed with IFA. 2 or 9 d after the second injection, lymph nodes were removed and cells were used for assays.

Proliferation Assay. Cells (4 × 10^5) were cultured in each well of a 96-well round-bottomed plate (Becton Dickinson) with no peptide or various concentrations of HA$_{306-318}$ or HA$_{109-120}$ or purified protein derivative (PPD; Connaught Laboratories Ltd.), at 37°C, 5% CO$_2$, for 72 h in RPMI 1640 (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL), 10 mM Hepes (GIBCO BRL), 50 U/ml penicillin/streptomycin (GIBCO BRL) and 50 mM 2-mercaptoethanol (Sigma-Aldrich). Each well was then pulsed with 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech). 18 h later, cells were harvested with a Packard Micromate cell harvester and the incorporated radioactivity was measured by a Packard Matrix 96 Direct b Counter.

Cytokine Assays. IL-2 release was measured with the IL-2–sensitive cell line CTLL-2 (American Type Culture Collection[ATCC]). Inguinal lymph node cells (4 × 10^5 cells per well) were cultured with 0, 0.1, or 1 μM HA$_{306-318}$ for 24 h. Cell-free culture supernatants were then collected, stored at −70°C, and thawed once. CTLL-2 cells (4 × 10^5 cells per well) were incubated at 37°C, with 5% CO$_2$ for 24 h in RPMI 1640 with supplements (10% FBS, 2 mM L-glutamine, 1.5 gram per liter sodium bicarbonate; Sigma-Aldrich), 10 mM Hepes plus 24-h culture supernatants, in culture wells of 96-well round-bottomed plates. Plates were then pulsed with 1 μCi of [^{3}H]thymidine for an additional 18 h, harvested, and counted. The level of IL-2 was calibrated against a standard curve of rIL-2. Cell-free culture supernatants were collected after 48 h and IFN-γ was measured by ELISA for mouse IFN-γ using Quantikine cytokine ELISA kit (R&D Systems) according to the manufacturer's protocol. All assays were performed in triplicate.

Flow Cytometric Analysis. Pooled lymph node and spleen cells (10^6) from various mice (adoptively transferred), 3 d after transfer of cells, 2, 3, or 5 wk after immunization were preincubated with Fc-γ receptor-blocking antibody 2.4G2 (HB-197, ATCC). Cells were washed and stained with biotinylated anticlonotypic TCR mAb (6.5, provided by H. von Boehmer, Harvard University, Boston, MA; reference 8) and avidin-cychrome (Cyc). The following mAbs were used for analysis: anti–mouse CD4-FITC; anti–mouse CD4-PE; anti–mouse CD62L-PE; anti–mouse CD45RB-PE; anti–mouse CD69-PE; anti–mouse CD25-FITC; and anti–mouse CD44-FITC. 48 h after the second peptide injection, 10^6 cells of recipient mice were stained with anticlonotypic TCR mAb-Cyc, anti–mouse CD4-FITC, and anti–mouse CTLA-4 PE. 9 d after the second peptide injections, 5 × 10^6 cells of HLA-DR1 Tg mice were cultured either with peptide (10 μM) alone or with rIL-2 (10 U/ml) for 8 d. Cells were stained with HA-DR1-SA-PE oligomers (provided by T. Cameron and L. Stern, MIT, Cambridge, MA) for 3.5 h at 37°C followed by anti–mouse CD4-FITC or anti–mouse CD4-Cyc and anti–mouse CTLA-4–FITC (Southern Biotechnology Associates, Inc.). All antibodies were purchased from BD PharMingen. The samples were analyzed on a FACScan™ by using CELLQuest™ software (Becton Dickinson). In each case, 5–10 × 10^4 events were collected.

In Vivo Antibody Treatments. HLA-DR1 Tg mice received intraperitoneal injections of 85 μg of hamster anti–mouse CTLA-4 mAb from the culture supernatant of the UC10-4F10-11 cell line (ATCC), which was purified on a protein A column in our laboratory and showed >95 purity by SDS-PAGE and silver staining. Injections were done on days 0, 1, 2, 3, 5, and 7 after the tolerogenic injection of various doses of HA$_{306-318}$ in IFA.

Mixing Experiments for Potential Presence of Immunoregulatory Cells in Anergic Groups. The regulatory capacity of anergic T cells was analyzed in a culture in vitro. Anergic cells (2 × 10^5) either from 0.0005 nmol or 0.05 nmol groups were incubated with 4 × 10^5 responder cells from each nonanergized group of HLA-DR1 Tg mice together with various concentrations of HA$_{306-318}$ (0, 0.1, 1, 10 μM). As positive controls, responder cells (0 and 5 nmol groups) were treated with rhTGF-β (0.1, 1, 10 ng/ml; R&D

Systems) for 48 h. Proliferative responses were examined by [³H]thymidine incorporation.

Results

Acquisition of Memory Phenotype in 6.5 TCR Tg Cells. The surface phenotype of naive T cells is relatively well characterized, and they express high levels of L-selectin (CD62L), CD45RB, and low levels of CD44. In contrast, the surface phenotype of memory T cells has remained controversial. It has been proposed that memory T cells express CD45RB^{lo}, CD62L^{lo}, and CD44^{hi}. In comparison to activated cells, memory T cells are presumed to be small and to have low expression of activation markers such as CD25 (IL-2R) and CD69 (9). Memory T cells migrate from blood and spleen to lymph nodes at a low rate (10,
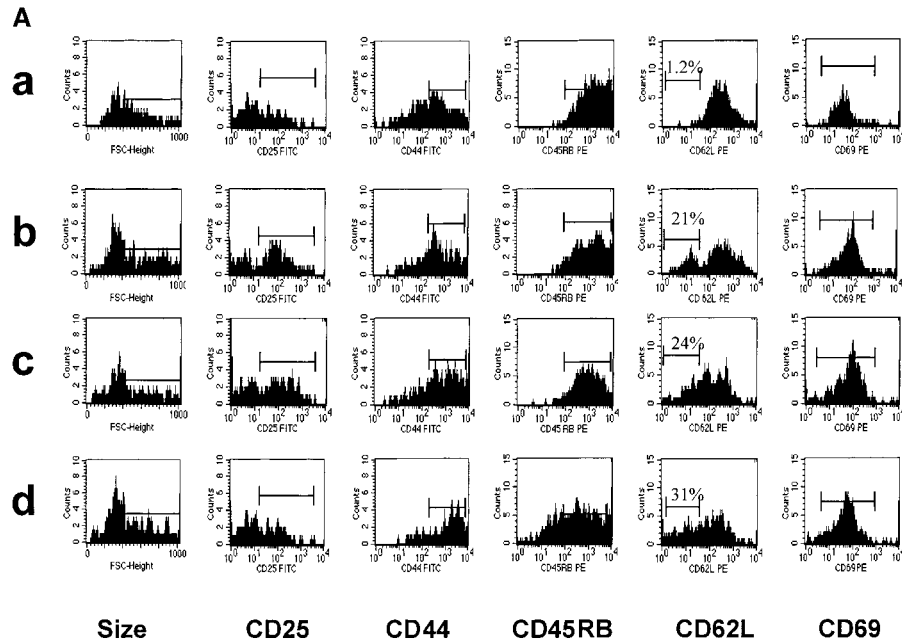
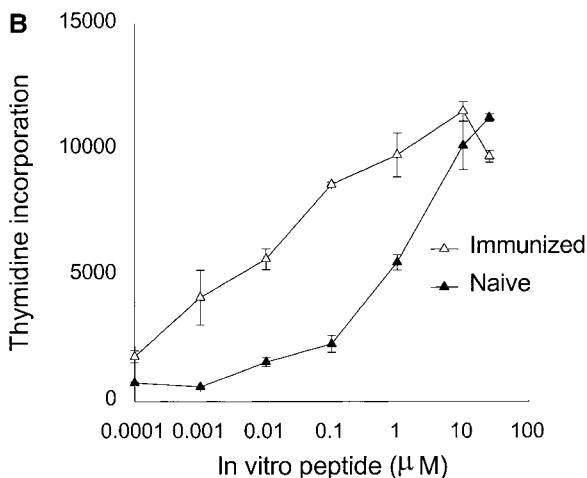|	Days after transfer	Days after immunization	Size	CD25	CD44	CD45RB	CD62L	CD69
a	3	0*	604	119	1391	2200	758	50
b	3	14	797	180	1576	1832	589	104
c	3	21	766	225	1760	1413	325	100
d	3	35	701	134	2356	1208	258	68

Mean Fluorescence Intensity, * No immunization

Figure 1. (A) Surface phenotypes of clonotypic HA-specific CD4 T cells in naive and immunized mice: splenocytes and lymph node cells were pooled and triple stained with anti-CD4, anticlonotypic TCR antibody (mAb 6.5), and indicated antibodies. Cell size and levels of expression of CD25, CD44, CD45RB, CD62L, and CD69 were measured by gating on the CD4+ and mAb 6.5+ population. (B) Memory cells respond faster and more efficiently to lower peptide doses than do naive cells; lymph node cells from adoptively transferred mice at 3 d (▲) and 5 wk after immunization with HA$_{109-120}$ (△) were prepared. Cells were restimulated with various concentration of HA$_{109-120}$ peptide. Proliferation assay was performed as described in Materials and Methods.

11), but reintroducing antigen can accelerate their recruitment back into lymph nodes (9, 11).

To trace specific T cells and to characterize their phenotype, we used 6.5 TCR Tg mice specific for complexes of $HA_{109-120}$-I-E^d (8). TCR Tg CD4$^+$ T cells were transferred into B10.D2 recipient mice, and 3 d later (to allow for homing of transferred cells to the lymphoid organs), recipients were immunized with 15 nmol of $HA_{109-120}$ in CFA. The group called "naive" did not receive any peptide. Lymph node and spleen cells were analyzed by size and three-color FACS$^{®}$ for expression of CD4, clonotypic TCR epitope, and levels of expression of memory markers. $HA_{109-120}$–specific CD4$^+$ T cells were stained for CD44, CD25, CD69, CD45RB, and CD62L on day 3 after transfer and 2, 3, and 5 wk after immunization. As seen in Fig. 1 A, during the 5 wk after immunization the expression level of CD44 increased, whereas levels of CD45RB, CD62L, CD25, and CD69 decreased. Moreover, the average cell size decreased during the 5-wk period, consistent with distinctive smaller size for memory versus activated T cells.

Memory T Cells Respond more efficiently to Lower Doses of Peptide. A more reliable marker for memory T cells is their characteristic higher sensitivity to antigenic stimulation compared with naive T cells (12). For further evaluation of the generation of memory T cells 5 wk after immunization, we compared the dose-proliferative response of cells from adoptively transferred mice 3 d after transfer (naive phenotype) and 5 wk after immunization (memory phenotype). Fig. 1 B shows that memory T cells proliferated in response to 100-fold lower peptide concentrations than did naive T cells.

Induction of Tolerance in TCR Tg Cells In Vivo. The above data strongly suggested that memory T cells developed 5 wk after immunization. Next, we examined whether low doses of agonist peptides could induce unresponsiveness in memory T cells in vivo. Induction of unresponsiveness occurred when low doses of peptide were administered 5 wk after priming (Fig. 2 A). Lack of vigorous proliferation in tolerized groups was not due to the deletion of antigen-specific cells, because we detected comparable percentages of CD4$^+$ 6.5 TCR$^+$ cells in all groups that ranged between 0.40–0.64% (0 nmol group, 0.40%; 0.5 nmol group, 0.44%; 10 nmol group, 0.64%).

Specificity of Tolerance Induced by Low Doses of Peptide. The mycobacterium debris antigens in CFA used in the first immunization could prime non-TCR Tg cells to respond to the bacterial PPD challenge in vitro. Thus, in order to control for specificity of the tolerance induced we tested the response levels of all groups to two different doses of PPD in vitro. The loss of proliferation of tolerized cells to $HA_{109-120}$ was clearly antigen specific as the proliferation to PPD was not altered significantly (Fig. 2 B).

Induction of Tolerance by Low Doses of $HA_{306-318}$ Peptide in HLA-DR1 Tg Mice. The above experiments demonstrated that tolerance can be induced among memory T cells of a single clonal origin adoptively transferred to a syngenic nonTg recipient. To further establish these findings and to test whether multiple T cell clones specific for the

same pair of peptide–MHC could also be tolerized by this treatment, we used Tg mice that express the human class II molecules, HLA-DR1. These Tg mice carry chimeric I-E/DR1 molecules where the peptide-binding groove is composed of HLA-DR1, but the membrane proximal domain is murine I-E to allow undisturbed interactions with murine CD4 on T cells. Importantly, these Tg mice develop a diverse T cell repertoire and can give rise to human DR–restricted immune responses after challenge with peptides that bind HLA-DR1 (13). In addition, our previous results demonstrating that anergy can be induced in T cell clones by low densities of peptide–MHC had used HLA-DR1 in complex with $HA_{306-318}$. Notably, interaction of HLA-DR1 and $HA_{306-318}$ peptide has been well characterized
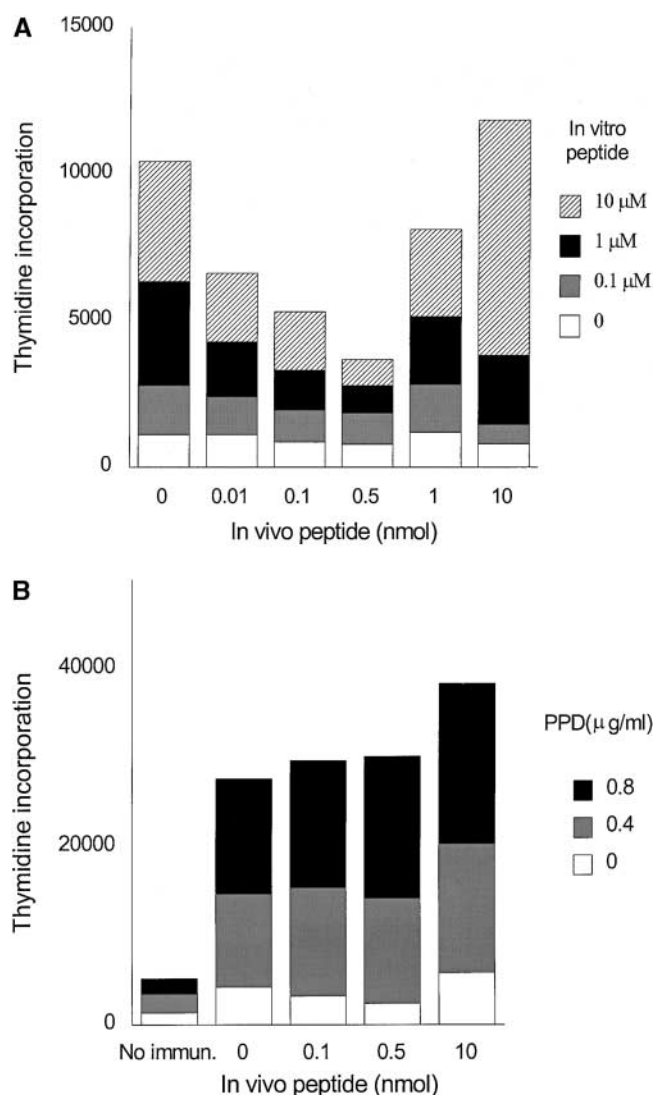

Figure 2. Induction of anergy in adoptively transferred mice with T cells from $HA_{109-120}$–specific TCR Tg donors. 3 d after adoptive transfer mice were immunized with $HA_{109-120}$ in CFA, 5 wk after they received different doses of $HA_{109-120}$ in IFA. 9 d later cells were harvested and cultured with different doses of $HA_{109-120}$ (A) and PPD (B) and proliferation was measured by [^{3}H]thymidine incorporation. Representative of five independent experiments.
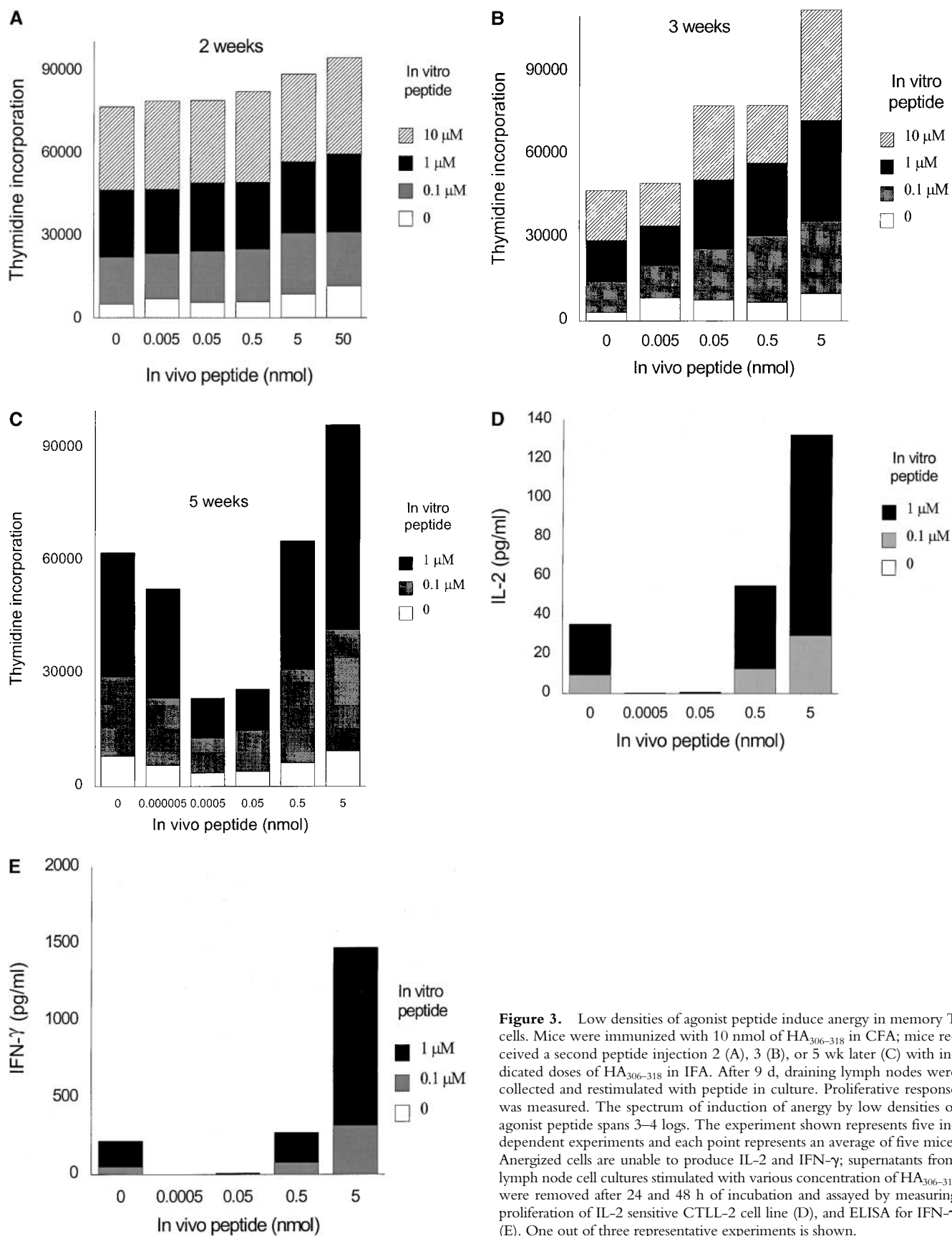
Figure 3. Low densities of agonist peptide induce anergy in memory T cells. Mice were immunized with 10 nmol of $HA_{306-318}$ in CFA; mice received a second peptide injection 2 (A), 3 (B), or 5 wk later (C) with indicated doses of $HA_{306-318}$ in IFA. After 9 d, draining lymph nodes were collected and restimulated with peptide in culture. Proliferative response was measured. The spectrum of induction of anergy by low densities of agonist peptide spans 3–4 logs. The experiment shown represents five independent experiments and each point represents an average of five mice. Anergized cells are unable to produce IL-2 and IFN-γ; supernatants from lymph node cell cultures stimulated with various concentration of $HA_{306-318}$ were removed after 24 and 48 h of incubation and assayed by measuring proliferation of IL-2 sensitive CTLL-2 cell line (D), and ELISA for IFN-γ (E). One out of three representative experiments is shown.

biochemically (14–16). Thus, testing HLA-DR1 mice had multiple advantages.

To generate memory T cells, an immunogenic dose (10 nmol ∼15 µg) of HA$_{306-318}$ in CFA was injected and different groups of mice received a second injection of variable doses of HA$_{306-318}$ 2, 3, or 5 wk later. We also tested 2 and 3 wk intervals because in vivo tracing of DR1/HA$_{306-318}$–specific T cells is not currently available (17, 18). Slightly lower doses of HA$_{306-318}$ were used to compensate for the higher binding affinity of HA$_{306-318}$ for DR1. HA$_{306-318}$-DR1 complex has a dissociation half-time of 6 d at 37°C (19). Cells from the draining nodes were removed and tested in a proliferation assay 9 d later. At short intervals between CFA priming and administration of peptide in IFA, no tolerogenic effects were observed (Fig. 3, A and B), consistent with the later development of memory phenotype. When low peptide doses were administered 5 wk after the initial priming we observed tolerance. Fig. 3 C depicts proliferation of cells from mice tolerized by low doses/densities of HA$_{306-318}$ (0.0005–0.05 nmol) in vivo. Cells from these groups proliferated significantly less well than cells from other groups that had received peptide doses <0.0005 nmol or higher than 0.05 nmol. The in vivo doses capable of inducing unresponsiveness spanned 3–4 logs. The inverse bell-shaped pattern of unresponsiveness to a range of peptide doses resembled T cell clones (7), where densities between 1–10 peptide–DR1 complexes per APC had the greatest inhibitory effects. Similar results were observed when the time required for induction of anergy was extended to 7 and 12 wk (see Fig. 4, A and B) as a further proof for the longevity of memory T cells and their susceptibility to tolerance induction by low avidity stimulation. Although in most experiments we have used draining lymph nodes isolated 9 d after tolerogenic peptide injec-

tion, tolerance was established at time points tested as early as 2 d after second peptide injection, and persisted up to 27 d, that latest time point tested (data not shown).

It has been proposed that T cell anergy is a consequence of the inability of a T cell to produce IL-2 (20). To determine the effect of various doses of HA$_{306-318}$ on IL-2 production, culture supernatants of draining lymph node cells were assayed for IL-2 release. Measurement of IL-2 levels showed that cells from mice exposed to 0.0005–0.05 nmol of HA$_{306-318}$ in vivo had markedly diminished IL-2 levels (Fig. 3 D). As shown in Fig. 3 E, production of IFN-γ was also significantly reduced in tolerized cells. The cytokine responses were antigen specific because cells cultured without peptide contained no detectable cytokines. It has been reported that low densities of antigen (21, 22) in IFA (23) may cause Th cells to differentiate into Th2 rather than Th1 cells. We examined cells for Th2 cytokines such as IL-4, IL-5, and IL-10 secretions in culture supernatants. We also analyzed intracellular IL-4 production in all groups. None of the assays used detected measurable levels of IL-4, IL-5, and IL-10 in any of the groups tested (data not shown). Furthermore, IFN-γ secreted in nontolerized groups (0, 0.5, 5 nmol) reflects their differentiation into Th1 cells (Fig. 3 E).

Tolerance Is not due to Suppressor T Cells or Deletion. A potential mechanism responsible for induction of tolerance in mature peripheral T cells is immunoregulation. Two models have been proposed to explain the effects of immunoregulatory T cells. One involves suppressive effects of T cell–derived cytokines, such as IL-4, IL-10, and TGF-β, which inhibit the activation of IL-2–producing T cells (24, 25), and the other postulates the suppressive effects based on competition with responding cells for access to APC surface antigens or costimulatory molecules (26). To deter-
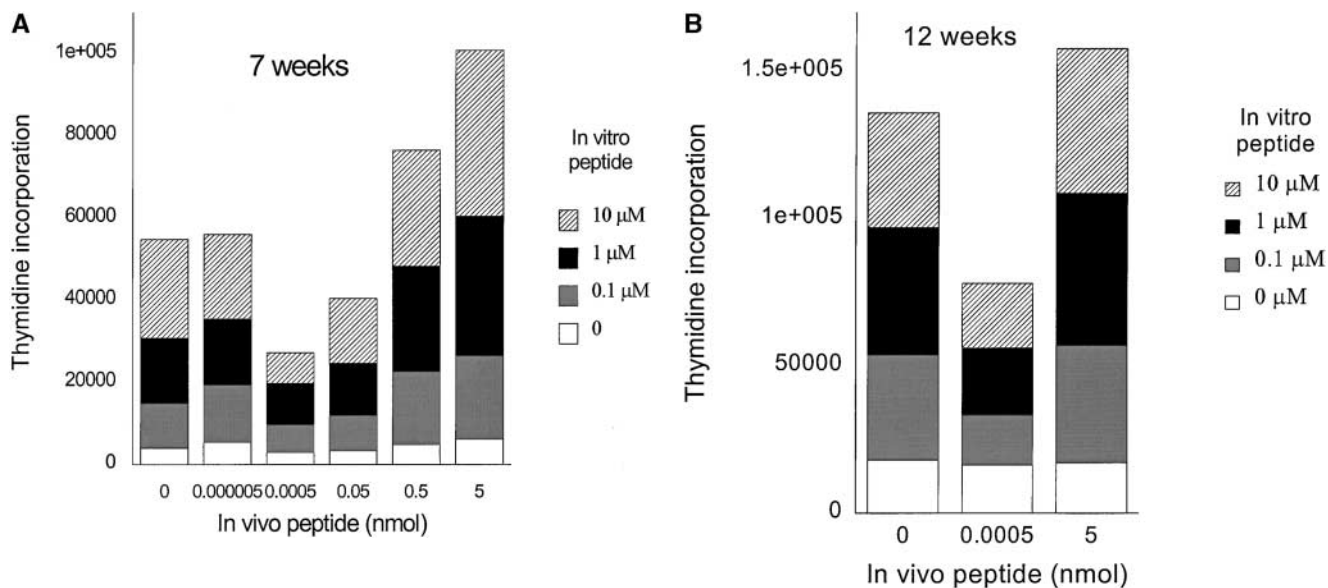

Figure 4. Memory T cells are targets for anergy. Seven (A) or 12 wk (B) after immunization with HA$_{306-318}$ in CFA, mice received a second peptide injection as described in Materials and Methods. The proliferative response to in vitro peptide restimulation was determined by [³H]thymidine incorporation. The experiment shown represents five independent experiments and each point represents an average of five mice.

mine whether tolerance was induced by suppressor T cells in our system, we mixed responder cells (nonanergized groups) with cells from two anergized groups (0.0005–0.05 nmol) in the presence of various concentrations of HA$_{306-318}$, and then tested for T cell proliferation (Fig. 5, A and B). To control for whether the responding cells were responsive to suppressor cytokines, responder cells (0 and 5 nmol groups) were treated with different doses of TGF-β in vitro (Fig. 5 C). Fig. 5 shows that tolerized cells were unable to inhibit proliferation of antigen-specific responder T cells, although TGF-β treatment had inhibitory effects.

Apoptosis (27) and deletion (28) are well-established mechanisms for peripheral tolerance. To determine whether tolerance was caused by deletion of the specific T cells, we used HA-DR1 oligomers to trace HA$_{306-318}$-specific T cells in DR1 Tg lymph node cells that had been tolerized. Similar to other preparations of oligomeric human class II (17), HA-DR1-SA-PE oligomer (18) failed to detect specific cells directly from a pool of lymphocytes.

Nonetheless, specific cells were detectable after 8 d in culture. We followed the protocol established previously (18) and detected specific T cells that were stained by HA-DR1-SA-PE oligomers in all groups of mice (Fig. 6). The percentages of positive cells were comparable in anergized and nonanergized groups, ranging from 0.7–1.3%, indicating that they were not deleted. It has been demonstrated in models in vitro (20) and in vivo (29) that anergic T cells are unable to divide because they do not produce IL-2 and that addition of exogenous IL-2 can prevent or reverse anergy (7, 30). Upon addition of peptide and IL-2, the percentage of positive cells in the anergized group (0.0005 nmol peptide in IFA) doubled, confirming persistence of anergic cells (Fig. 6). The background counts for unprimed naive cells were 0.09%.

These experiments rule out suppression or deletion as an explanation for tolerance in our system, but provide clear evidence for anergy as the most likely explanation.
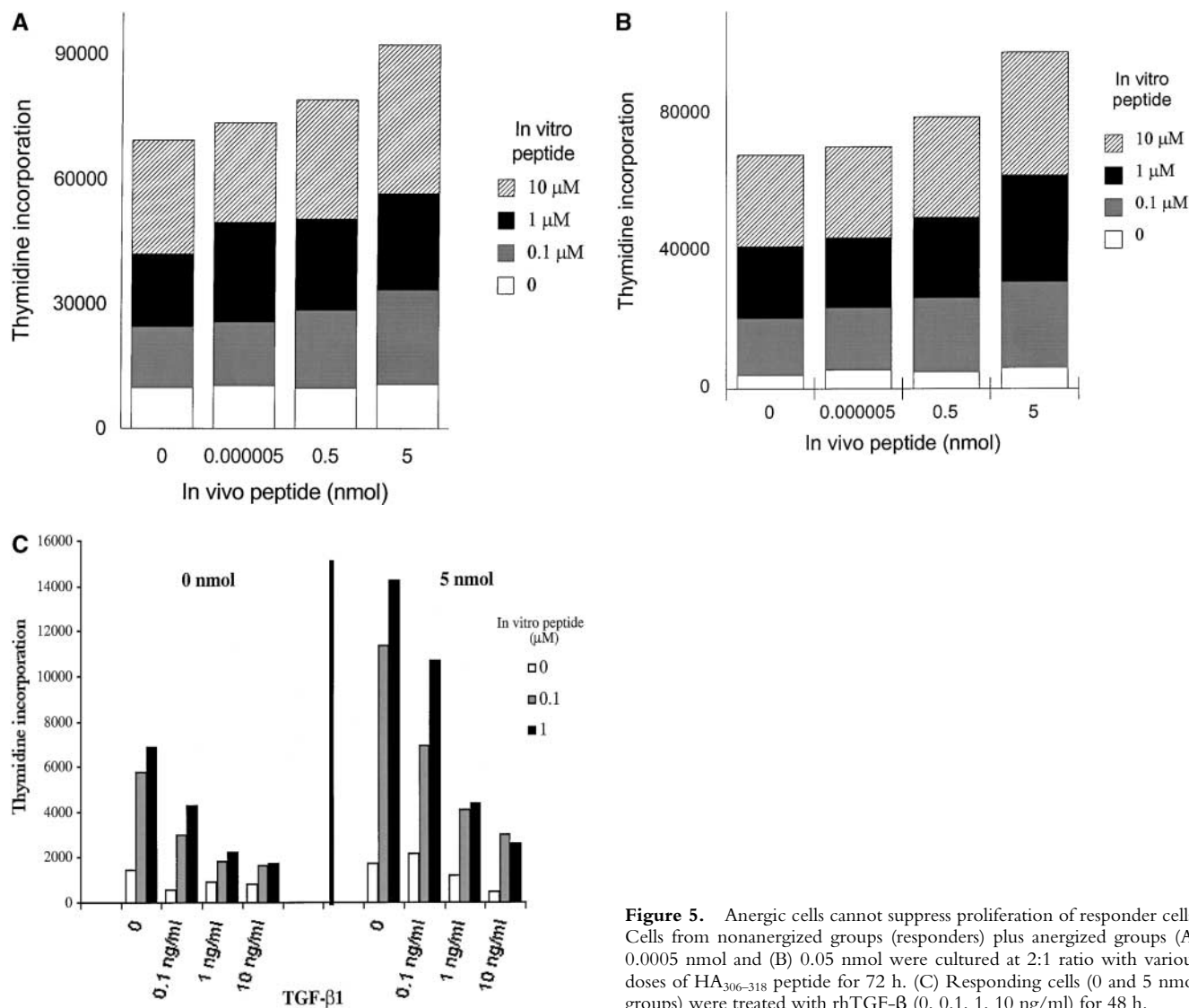

Figure 5. Anergic cells cannot suppress proliferation of responder cells. Cells from nonanergized groups (responders) plus anergized groups (A) 0.0005 nmol and (B) 0.05 nmol were cultured at 2:1 ratio with various doses of HA$_{306-318}$ peptide for 72 h. (C) Responding cells (0 and 5 nmol groups) were treated with rhTGF-β (0, 0.1, 1, 10 ng/ml) for 48 h.
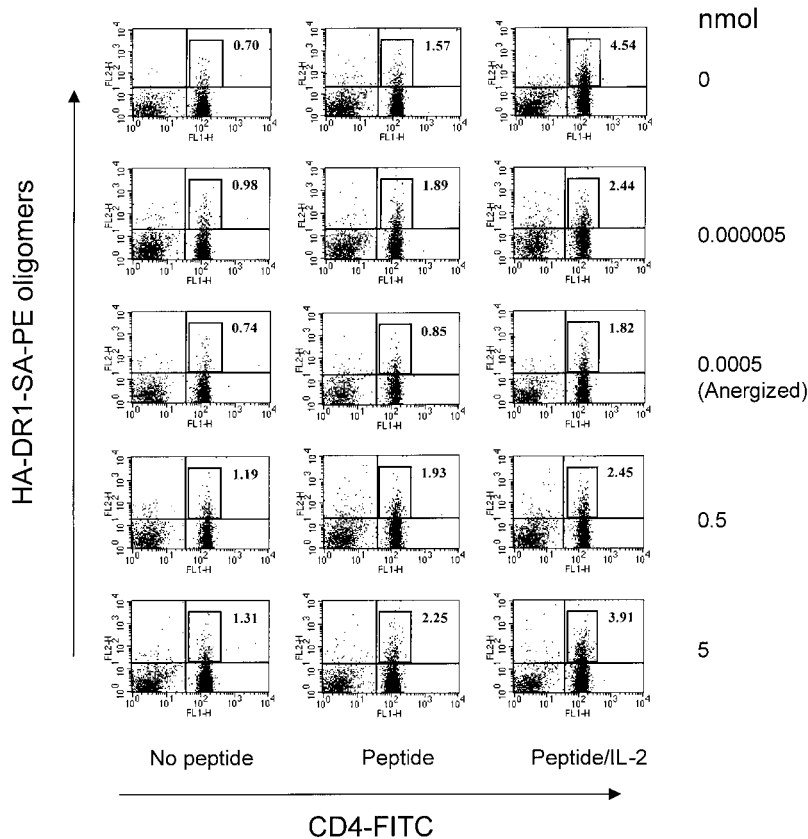
Figure 6. Anergized cells are able to expand in the presence of rIL-2. Splenocytes and lymph node cells from anergized and nonanergized mice were cultured without peptide, with peptide (10 μM) alone or with rIL-2 (10 U/ml) for 8 d. Cells were stained with HA-DR1-SA-PE oligomers and anti–CD4–FITC.

Upregulation of CTLA-4 Expression by Tolerogenic Doses of HA Peptides. A critical role for CTLA-4 in negative regulation of the immune response has been established by the findings that CTLA-4–deficient (CTLA$^{-/-}$) mice display a severe lymphoproliferative disorder combined with massive lymphocytic infiltration that leads to tissue destruction and death of the mice at 3–4 wk of age (31, 32). T cell tolerance in vivo may arise from dominant engagement of B7 molecules by the CTLA-4 over CD28 and not from lack of costimulation (33). To test possible involvement of CTLA-4 we measured levels of CTLA-4 expression in clonotypic HA-specific CD4$^+$ T cells (6.5$^+$, CD4$^+$) of draining lymph nodes 2 d after the second peptide injections (34, 35), and in HA$_{306–318}$–specific CD4$^+$ T cells from HLA-DR1 mice, 8 d after in vitro culture in the presence of peptide. Interestingly, we observed a direct correlation between the levels of CTLA-4 expression and the induction of T cell unresponsiveness (Fig. 7, A and B). Mice exposed to tolerogenic dose of peptide expressed an increased level of CTLA-4 on their T cells, whereas T cells from mock-immunized mice and those injected with no peptide or an immunogenic dose had comparable levels of CTLA-4. These observations suggest that tolerance induced by low densities of agonist peptide in vivo may occur through CTLA-4 signaling.

Blocking CTLA-4 Prevents Induction of Tolerance by Low Density of Agonist Peptide. To ascertain the regulatory role of CTLA-4 in induction of tolerance in this system, HLA-DR1 Tg mice were treated with anti–CTLA-4 mAb. Im-
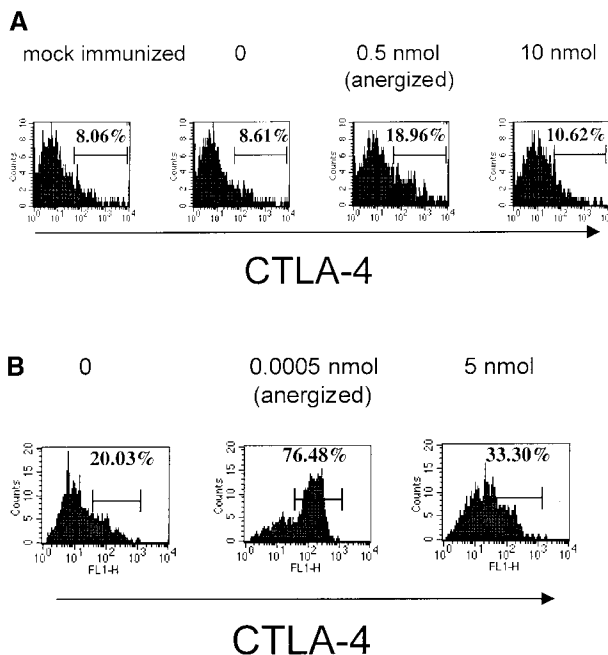

Figure 7. Expression of CTLA-4 on cells exhibiting anergy. Surface expression of CTLA-4 on (A) clonotypic HA-specific CD4 cells from draining lymph nodes of adoptively transferred mice 48 h after the tolerogenic peptide administration and (B) HA$_{306–318}$–specific CD4$^+$ T cells from HLA-DR1 mice 8 d after in vitro culture in presence of peptide as examined by anti–CTLA-4 antibody staining. Results are from one out of two independent experiments.

munized mice were treated with multiple injections of anti–CTLA-4 mAb (intraperitoneally) on days 0, 1, 2, 3, 5, and 7 after injection of tolerogenic peptide. Draining lymph nodes were removed on day 9 and cell proliferation to in vitro peptide restimulation was tested. Fig. 8 shows that lymph node cells from mice given a tolerogenic peptide dose proliferated weakly. In contrast, cells from mice treated with anti–CTLA-4 mAb showed a strong proliferative response. Animals injected with 0 or 5 nmol of $HA_{306-318}$ in the presence or absence of anti–CTLA-4 mAb showed similar levels of proliferation. Therefore blocking of CTLA-4 prevents transmission of signaling required for T cell tolerance induced by low density of agonist peptide. These results demonstrate that induction of tolerance in memory T cells by low density peptide presentation is accomplished by the expression of CTLA-4.

Discussion

Low Densities of Agonist Peptides Induce Anergy In Vivo. We have recently described a novel mechanism for induction of a long-term T cell anergy by presentation of low densities of agonist peptide–MHC in CD4$^+$ T cell clones. It was estimated that 1–10 agonist $HA_{306-318}$ in complex with HLA-DR1 could internalize <1,000 TCR (7). This was in sharp contrast to altered peptide ligand effects that are generally observed upon presentation of 2–3 orders of magnitude higher densities of peptide–MHC. The anergic cells were unable to proliferate, secretion of IL-2 was inhibited, and secretion of IFN-γ was reduced. Nevertheless, no inhibitory effects were observed on downregulation of TCR or upregulation of IL-2R. Such a phenotype denotes a state of partial stimulation of T cells similar to that defined for the effects of classic antagonist peptides (36, 37) or TCR engagement in the absence of signal 2 (38). In this report, we address the relationship between the density of peptide–MHC complexes and the differential transduction of signals in T cells in vivo.

In this study, we provide evidence that low-avidity engagement of T cells, by low densities of agonist peptide–MHC, leads to the induction of T cell unresponsiveness in the periphery in vivo. High-avidity engagement of TCR by peptide–MHC may lead to formation of immunological synapse (39, 40) and sustained signaling (41). One can assume that low-avidity engagements might interrupt cytoskeletal rearrangements and full recruitment of necessary signaling molecules to the plasma membrane, resulting in negative signaling and T cell anergy. However, the detailed mechanism of these events remains to be tested.

Memory T Cells as Targets for Anergy Induction by a Low Density of Peptide. Although induction of anergy among naive T cells has been readily generated (42, 43), induction of anergy in memory T cells in vivo has proved to be difficult (3). Qualitative changes that distinguish memory from naive T cells render memory T cells able to respond to antigenic challenge faster and more efficiently than naive T cells (9, 12). Multiple reasons could account for this effect:
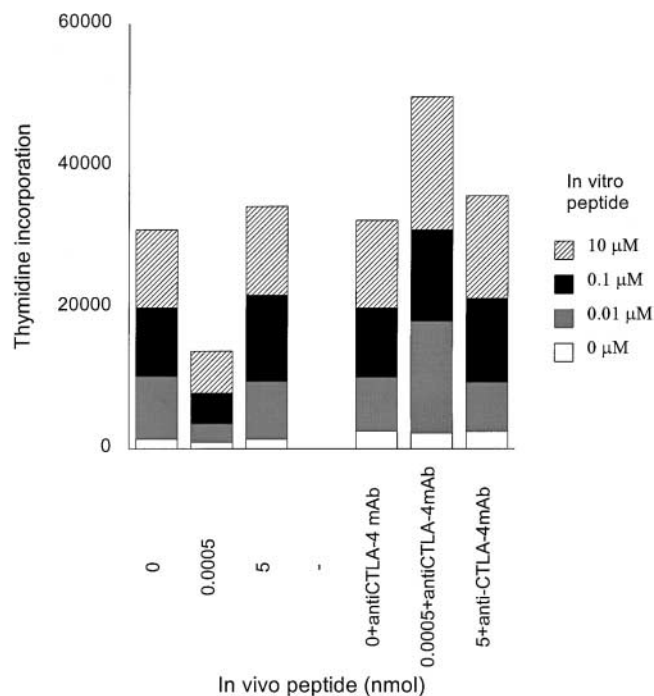

Figure 8. Blocking of CTLA-4 engagement prevents induction of anergy. HLA-DR1 Tg mice were immunized and injected with $HA_{306-318}$ as described in Materials and Methods. One group of these mice was treated (intraperitoneally) with multiple anti–CTLA-4 mAb injections on days 0,1, 2, 3, 5, and 7 after the second peptide injection with various doses of $HA_{306-318}$.

differences in activation requirements, TCR signaling, expression of adhesion molecules (9), and dependence on costimulatory signals for activation between memory and naive T cells (3, 44). In addition, memory CD8 T cells have been shown to have a distribution of Lck different from that of naive T cells. Lck seems to be associated with CD8 in memory, but evenly distributed in naive T cells (45).

Several observations in this study support the notion that low densities of peptide–MHC target memory T cells. First, and most critical, is the long generation time, a minimum of 5 wk (46–48), for T cells to become receptive to anergy. Second, specific memory T cells were able to proliferate at lower peptide doses than did naive cells (Fig. 1 B), consistent with the observations of Rogers et al. (12). Third, in comparison to cells from nonimmunized mice, primed cells expressed low levels of CD62L and CD45RB and increased levels of CD44 (Fig. 1 A). Finally, increased expression of CTLA-4 also correlated with induction of anergy (Fig. 7, A and B), consistent with the finding that memory and not naive T cells express intracellular stores of CTLA-4 (6, 49).

It seems reasonable to assume that memory T cells would respond to low densities of peptide–MHC because of the specific signaling machinery that is already associated with their membranes. For naive T cells, such low densities of ligands might be ignored.

Previous studies on peripheral tolerance attributed the loss of T cell function to different mechanisms (24, 50–52).

Diminished proliferation marks the acquired state of un-responsiveness (anergy) in our system. Lack of proliferation in cells exposed to low doses of peptide was not due to cellular death for the following reasons: first, antigen-specific T cells were detectable in lymph node cells of anergized mice (Figs. 6 and 7); second, anti–CTLA-4 treatment restored vigorous proliferation of anergized cells (Fig. 8); and third, anergized HA-specific CD4$^+$ T cells from HLA-DR1 Tg mice proliferated in the presence of rIL-2 (Fig. 6). Our conclusions are supported by reports demonstrating that CTLA-4 ligation does not induce apoptosis (53, 54) and that memory T cells are more resistant to apoptosis (44, 55).

Recent reports suggest a role for immunoregulatory CD25$^+$CD4$^+$ T cells in the control of tolerance (56). Immunoregulatory T cells are present in the subpopulation of CD4$^+$ cells that express activation/memory markers CD25$^+$CD45RB^{low} (57). Another report suggests that the CD4$^+$CD25$^+$ population contains a significant proportion of cells with a naive/resting phenotype (58) that seem to express CTLA-4 constitutively and to suppress the activation and proliferation of other T cells, by competing for costimulatory molecules (59) or by triggering TGF-β secretion (60). Nevertheless, cells rendered anergic by low densities of antigen did not show any suppressive function: they did not cause neither specific inhibition of responder T cell proliferation (Fig. 5, A and B) nor did they induce a general suppression in response to PPD (Fig. 2 B).

Lack of proliferation and production of IL-2 and IFN-γ in anergized groups is less likely to be due to a Th2 switch. Two important observations support this notion: first, that no IL-4, IL-5, or IL-10 production was detected; and second, CTLA-4 expression, which was upregulated in the anergized group, is reported to limit Th2 differentiation (61).

Finally, ignorance has been evoked as a mechanism for T cell tolerance (62). The tolerance described here cannot be explained by ignorance because tolerized cells had to be primed and responded fully to the peptide challenge in the absence of a second low dose treatment. Thus, taken together, T cell anergy best describes our findings.

Multiple reports indicate that resting B cells may be the predominant APCs that induce tolerance. The tolerogenicity of resting B cells may be due to the absence of costimulatory molecules such as B7, which is highly expressed in DCs and activated B cells (63). It has been shown, however, that both activated and resting B cells can induce tolerance in naive but not memory T cells (2), which may be due to less stringent requirement of memory T cells for costimulatory molecules. All APCs, including resting B cells can stimulate memory T cells (5). Although a role for DCs as the most potent antigen presenters has well been documented, its role as a tolerogen is less clear. However, under the experimental conditions used here, it is likely that DCs that express high levels of MHC class II, may preferentially bind peptides offered at the tolerogenic, lower doses. At higher peptide doses all APCs might access and present antigenic peptides. Notably, some MHC class II expressed on DCs are shown to be empty (64), which provokes the thought of a new role for DCs as inducers of tolerance in peripheral T cells (65). Studies on the way address these issues.

Use of two different Tg mouse models provides clear evidence that a monoclonal 6.5$^+$ TCR Tg mice or multiclonal T cell populations specific for HA$_{306-318}$ in HLA-DR1 Tg mice, can be rendered tolerant. Other reports have also investigated differences in a similar Tg system, documenting that in the non-TCR Tg mouse model, T cell clones of different affinities for a given peptide–MHC complex can be stimulated or tolerized (66).

CTLA-4 Mediates Anergy. Manifestation of anergy in this system correlates with the upregulation of CTLA-4 which can be inhibited by administration of anti–CTLA-4 mAb in vivo. Several groups have proposed that induction of peripheral T cell tolerance is a direct consequence of CTLA-4 engagement by B7 (33, 67). CTLA-4 can inhibit T cell responses extracellularly, by competing for B7 ligands (68), or intracellularly, by recruiting src homology 2 domain–containing protein tyrosin phosphatase (SHP)-2 and inhibiting tyrosine phosphorylation (69), by triggering TGF-β production (70), or by blocking cell cycle progression at the late G1 to S phase (1). Alternatively, full inhibitory function of CTLA-4 may require both extra and intracellular domains (71). We observed an increased level of CTLA-4 in cells that exhibited anergy compared with other groups of cells that received doses below or above the tolerogenic dose of peptide (Fig. 7). Strong evidence for the role of CTLA-4 in the induction of anergy is provided in Fig. 8, in which enhanced T cell proliferation of the anergized cells of mice that were treated with anti–CTLA-4 blocking antibody is observed. Lack of any measurable effect on proliferation of cells from other groups that did not express CTLA-4 indicates the specific and controlled effects of anti–CTLA-4 antibody. In most reports an increase in proliferation correlates with parallel increase in IL-2 and IFN-γ secretion (1, 33, 61, 72, 73). However, it is of interest to consider the possibility that CTLA-4 blockade may affect the sensitivity of T cells to TCR ligand and possible effects on the proportion of cytokine-producing cells in favor of specific cytokines (74). Future experiments will address these issues.

Immediate implications of T cell anergy by a low density of agonist peptide–MHC include autoimmunity and viral and tumor surveillance. Memory T cells, specific for self, might need to be kept tolerized by encountering low densities of peptide–MHC, which could be an important process in the prevention of organ-specific autoimmune diseases. Furthermore, understanding of T cell responses to presentation of low densities of agonist peptide–MHC in vivo is of great significance because of its pertinence in viral infections and in antitumor responses. Many viruses and several tumors are known to decrease expression of cell surface MHC class II (75). Alternatively, some tumor-associated peptides bind MHC poorly (76). The reduced surface expression of MHC and low affinity peptide–MHC complexes lead to presentation of low densities of specific peptide–MHC, which will induce anergy to the viral peptides or to tumor antigens through a CTLA-4–dependent

mechanism. A recent report demonstrating that CTLA-4 blockade reverses CD8^{+} T cell tolerance to tumor by a CD4^{+} T cell– and IL-2–dependent mechanism supports this notion (77). Thus, our findings demand a revision in design of strategies to overcome viral infections or tumor therapy. Rather than enhancing antigen presentation, one may have to focus on the reversal of anergy.

In conclusion, our results strongly suggest that low densities of agonist peptide induce anergy in memory T cells and that CTLA-4 is the dominant regulatory mechanism.

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