CD4⁺ T Cells Pass Through an Effector Phase During the Process of In Vivo Tolerance Induction


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CD4⁺ T Cells Pass Through an Effector Phase During the Process of In Vivo Tolerance Induction


An important process in the generation of tolerance to peripheral self-Ags is the induction of unresponsiveness in mature specific T cells. Although the end stage of this process, termed anergy, is well defined, the pathway by which naive T cells become anergic remains to be elucidated. Using an in vivo self-tolerance model, we demonstrate that CD4⁺ T cells pass through a significant effector stage on their way to an anergic state. This stage is characterized by production of effector cytokines, provision of help for CD8⁺ T cells, and induction of in vivo pathology within organs that express cognate Ag. These results suggest that the initial activation stage in T cell tolerance is similar to that seen in memory induction. They also suggest that autoimmune pathology can result during the natural process of tolerance induction rather than requiring that tolerance be broken. The Journal of Immunology, 2003, 170: 3945–3953.

Upon encountering their cognate peptide epitopes presented by professional APCs in secondary lymphoid organs, mature naive T cells can differentiate along one of two pathways. In the case of pathogen-derived epitopes, naive T cells typically differentiate into effectors that can participate in the neutralization of the pathogen and also differentiate into memory cells that can be efficiently reactivated upon secondary Ag exposure. In contrast, naive T cells with specificity for self-determinants become tolerated such that they lose the potential to express effector functions and to respond to secondary Ag stimulation. T cell tolerance is an important process of self/non-self discrimination for immunity to foreign as well as self- or tumor Ags. Understanding tolerance mechanisms is critical to manipulating autoimmunity and immunity to tumors or chronic infectious agents.

It has been well established that naive T cells receiving immunogenic stimulation undergo multiple rounds of division during which time they develop the ability to express effector functions, e.g., cytokine production, etc. (reviewed in Ref. 1). Interestingly, numerous in vivo models have indicated that naive T cells receiving a tolerogenic stimulus can undergo an initial proliferative phase before reaching a tolerant state (2–5). It has not been clear, however, whether T cells that are undergoing tolerance induction can demonstrate effector function as do their counterparts that have received immunogenic stimuli, or whether this initial proliferative phase is unassociated with effector function.

Using a transgenic model system that we have previously established to study CD4⁺ T cell peripheral tolerance induction to self-Ags (5, 6), we provide evidence that a population of adoptively transferred naive clonotypic TCR transgenic CD4⁺ T cells encountering their cognate self-Ag do demonstrate transient effector function before reaching a tolerant state. These tolerizing CD4⁺ T cells manifest effector function by three separate parameters: the ability to produce the effector cytokine IFN-γ upon Ag restimulation; the provision of help to CD8⁺ T cells; and the induction of inflammatory autoimmune pathology in an organ expressing the cognate Ag. These results indicate that immunogenic and tolerogenic T cell differentiation are more similar than previously thought, because both involve not only multiple rounds of proliferation but also the development of effector function. Thus, the main difference between the two pathways is the ability of immunized T cells to maintain effector function.

Materials and Methods

Transgenic mice

The C3-HA expressing transgenic mice have been previously described (6) (5). In short, the hemagglutinin (HA)² gene derived from the influenza virus A/PR/8/34 (Mount Sinai stain) has been placed under the control of the rat C3(1) promoter. Two founder lines were established in the B10.D2 genetic background. These two founder lines, C3-HAhigh and C3-HAlow, which contain 30–50 and 3 transgene copies, respectively, express the C3-HA hybrid mRNA in the same set of nonlymphoid tissues including the lung and prostate (6). Although the difference in total HA protein expression between C3-HAhigh and C3-HAlow was not directly measured, in the lung and prostate the difference is roughly 1000-fold as shown by bioassay of tissue extract induced hybridoma cytokine release (5).

The TCR-transgenic mouse line 6.5, which expresses a TCR recognizing an I-Ek-restricted HA epitope (130SFERFEFPKE[130]) generously provided by H. Von Boehmer, Harvard University, Boston, MA), was backcrossed nine generations onto the B10.D2 genetic background. The other TCR-transgenic mouse line Clone-4, which expresses a TCR recognizing a Kk-restricted HA epitope (5191YSTVASSL[526]) generously provided by L. Sherman, The Scripps Research Institute, La Jolla, CA), was backcrossed also for more than nine generations onto the Thy-1.1/1.1 B10.D2 genetic background. Because no clonotypic Ab is available for Clone-4 TCR, we use Thy-1.1 as a surrogate marker. Following adoptive transfer into Thy-1.2/1.2 recipients, we can assume that all the Thy-1.1⁺ CD8⁺ T cells

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²Abbreviations used in this paper: HA, hemagglutinin; NT, nontransgenic; vacc-HA, recombinant vaccinia virus expressing HA; RAG, recombination-activating gene; AIDN, activation-induced nonresponsiveness.

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express the HA-specific clonotypic TCR, because nearly all of the mature CD8+ T cells in the Clone-4 mice directly recognize the Kα-restricted HA epitope (7).

Transgenic mice were used for experiments between the ages of 8–24 wk. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Adoptive transfer

Clonotypic CD4+ or CD8+ T cells were prepared from pooled spleens and LNs of 6.5 or Clone-4 transgenic mice. Clonotypic percentage was determined by flow cytometry analysis. The activation marker CD44 was analyzed to ensure that these clonotypic cells were not activated in donor mice and were naive in phenotype. After three washings with HBSS, an appropriate number of cells were resuspended in 0.2 ml of HBSS for i.v. injection through the tail vein.

Extraction of lung-infiltrating lymphocytes

The lung was first minced by a surgical scalpel. Minced tissue was then digested with tissue digestion buffer (RPMI medium with 0.0025% hyaluronidase, 0.002% DNase, and 0.1% collagenase IV). Digestion was performed with gentle shaking at 37°C for 4–6 h. After passing through nylon mesh, the cell suspension was separated with Ficoll (Pharmacia, Peapack, NJ), and the buffy coat was collected.

Abs and flow cytometry

mAbs used for staining were: biotinylated anti-clonotypic TCR 6.5 (provided by H. Von Boehmer); avidin-PE (Caltag, Burlingame, CA); CyChrome-conjugated anti-CD4 (RM4-5), CyChrome-conjugated anti-CD8 (55-67), PE-conjugated anti-Thy-1.1 (OX-7), FITC-conjugated anti-CD44 (1 M7), biotin-conjugated B-220 (RA3-6B2), biotin-conjugated CD11c (HL-3), and biotin-conjugated NK1.1 (PK136) (all purchased from BD Pharmingen, San Diego, CA). FACS (BD Biosciences, San Jose, CA) was used for flow cytometry event collection, and events were analyzed using CellQuest software (BD Biosciences).

CD8+ T cell depletion

Dynabeads M-280 streptavidin (100–150 × 106; Dynal, Oslo, Norway) were used for depletion of CD8+ T cells from 100 × 10^6 splenocytes or LN cells. There were at least 4 beads per CD8+ T cell to be depleted. The magnetic beads were first washed once with Dynabeads Washing Buffer (1× PBS with 1% heat-inactivated FBS; Dynal). Splenocytes or LN cells were resuspended as 50 × 10^6/ml in Dynabeads Washing Buffer, and 10 μl of 0.5-mg/ml stock of biotinylated anti-CD8+ Ab (Ly-2, 55-67; BD Pharmingen) were added for every 100 × 10^6 cells. Cells were incubated with Ab on a gently rocking rotator at 4°C for 30 min before two washings with Dynabeads Washing Buffer. Then Ab-treated cells were resuspended with beads at a concentration of 50 × 10^6 cells/ml. They were incubated again on a gently rocking rotator at 4°C for 30 min. Beads and bound cells were removed with a magnetic rack as all cells resuspended in a 15-ml conical tube with a final volume of 4–6 ml. Cell suspension free of magnetic beads was collected. This was repeated once to remove all beads in suspension completely. CD8+ T cell-depleted cells were then washed three times with HBSS and counted before resuspended into an appropriate volume for injection.

Proliferation assay

A total of 1.5 × 10^6 splenocytes or LN cells collected from transfer recipients were incubated in round-bottom 96-well tissue culture plates with various concentrations of indicated synthetic HA peptide, either HA class II (110SFEREFIPKE219) or for CD4+ 6.5 or HA class I(318IYSTVASSLS236) for CD8+ Clone-4. From 24 to 72 h later, as indicated, cultures were pulsed with 1 μCi [3H]thymidine and incubated for an additional 16 h before harvest with a Packard Micromat cell harvester (Packard Bioscience, Meriden, CT). Determination of the amount of incorporated radioactive counts was performed with a Packard Matrix 96 direct beta counter (Packard Bioscience).

ELISA for cytokine release

IL-2 and IFN-γ were measured using ELISA kits purchased from Endogen (Woburn, MA), according to manufacturer’s instructions. Samples were the supernatant of 48 h in vitro cultures.

Results

Mice expressing high levels of a model self-Ag develop autoimmune pathology on receiving adoptive transfers of cognate naive CD4+ T cells

We have previously developed a model system for studying peripheral tolerance induction in CD4+ T cells, in which naive 6.5 TCR-transgenic CD4+ T cells specific for an I-Ealpha-restricted epitope from the influenza virus (PR8 strain) HA protein (8) are adoptively transferred into transgenic mice expressing HA as peripheral self-Ag (5, 6). The transgenic recipients express HA under the control of the rat C3(1) promoter, which directs HA expression to a number of parenchymal tissues, of which the lung expresses the highest levels. Two separate founder lines of C3-HA mice have been studied that express HA in the same subset of tissues but differ in the level of HA expression by at least three orders of magnitude (i.e., C3-HAhigh and C3-HAlow).

When spleen and LN preparations containing 2.5 × 10^6 naive 6.5 clonotypic CD4+ T cells were transferred into C3-HAlow mice, these 6.5 cells become anergic 7 days after transfer (6). C3-HAlow recipients tolerated transfer well and remained healthy. To our surprise, however, if C3-HAhigh mice were given the same number of naive 6.5 cells, they invariably died between 5 and 7 days after transfer. Pathological examination revealed that these animals displayed severe inflammatory changes in the lungs, with hepatization indicative of severe pneumonitis (Fig. 1A). Microscopically, there were dense inflammatory cell infiltrates around the pulmonary vessels. This vesicular cuffing was accompanied by endothelial changes suggestive of pulmonary vasculitis (Fig. 1B, top). A number of controls indicated that this pneumonitis was Ag specific. Neither transfer of nontransgenic (NT) B10.D2 spleen and LN preparations into C3-HAlow recipients nor 6.5 transgenic spleen and LN preparations into NT B10.D2 mice caused pneumonitis. Furthermore, to determine whether pneumonitis required HA to be expressed in the lungs, 6.5 transgenic T cells were primed in NT recipients by inoculation with 1 × 10^7 PFU of recombinant vaccinia virus expressing HA (vacc-HA). Although these NT recipients did not express HA in their lungs, there was an increase in the cellularity of the lung parenchyma (Fig. 1B, bottom), consistent with recent reports that primed T cells migrate to the lung in an apparently Ag-nonspecific manner (9, 10). However, there was minimal inflammation around the pulmonary vessels, and the recipients remained healthy, indicating that the pulmonary vasculitis in our C3-HAhigh recipient mice required HA expression in the lung.

The mortality of the C3-HAhigh recipient mice induced by adoptive transfer was dependent on the number of clonotypic T cells transferred into the C3-HAhigh mice. Whereas 100% mortality was observed in mice receiving spleen and LN preparations containing 2.5 × 10^6 clonotypic CD4+ T cells, 50% mortality was induced by transfer of 2.5 × 10^5 clonotypic cells, and mortality was not observed when the dose was further reduced. The decrease in mortality associated with transfer of fewer clonotypic cells was paralleled by a reduction in the number of clonotypic cells that could be extracted from the lung and the secondary lymphoid organs (Fig. 2A), as well as a lessened severity in pneumonitis as assessed at the histological level (Fig. 2B). However, with C3-HAlow mice as recipients of 6.5 spleen and LN preparations containing 2.5 × 10^6 clonotypic CD4+ T cells, no pathology was induced in the lung, and recipient mice remained healthy with T cell adoptive transfer.

To further characterize the lung inflammatory infiltrates in the C3-HAhigh mice, pulmonary lymphocytes were extracted for FACS analysis. The majority of lung infiltrating CD4+ T cells in
the C3-HA<sup>high</sup> recipients were in fact HA specific (i.e., 6.5<sup>+</sup>) (Fig. 3). These clonotypic CD4<sup>+</sup> T cells appeared to be in an activated state in that they were blasting and uniformly expressed high levels of CD44 (data not shown). There did not appear to be an increase in the numbers of B cells, NK cells, macrophages, neutrophils, or eosinophils (data not shown). Consistent with the histological data, the frequency of lung-infiltrating clonotypic CD4<sup>+</sup> T cells was higher in C3-HA<sup>high</sup> than in NT + vacc-HA recipients (Fig. 3).

In addition to the clonotypic CD4<sup>+</sup> T cells, the C3-HA<sup>high</sup> lungs contained a significant number of CD4<sup>+</sup> clonotypic cells (Fig. 3). Because it has previously been shown that the class II-restricted 6.5 TCR is selected on both CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells (8), it is not a surprise that these CD4<sup>+</sup>-clonotypic cells were CD8<sup>+</sup> T cells. Because autoreactive naïve class I-restricted CD8<sup>+</sup> T cells induce autoimmunity in an analogous system (11), it was of interest to determine the relative contribution of the clonotypic class II-restricted CD8<sup>+</sup> T cells to the etiology of autoimmune pneumonitis in our system. To address this issue, 6.5 spleen and LN preparations that had been depleted of CD8<sup>+</sup> T cells and contained 1 × 10<sup>6</sup> naïve clonotypic CD4<sup>+</sup> T cells were adoptively transferred into C3-HA<sup>high</sup> recipients that either contained an intact T cell repertoire or had been depleted of endogenous CD8<sup>+</sup> T cells using an in vivo depleting mAb. In both sets of recipients, pneumonitis developed as assessed by histological analysis (data not shown). However, each group exhibited a 50% reduction in mortality (two of four died in each group). Thus, although the clonotypic CD8<sup>+</sup> T cells appeared to contribute to the development of autoimmune pneumonitis, the clonotypic CD4<sup>+</sup> T cells were sufficient to mediate this effect.

**Clonotypic CD4<sup>+</sup> T cells become tolerized despite mediating autoimmune pathology**

Because the adoptively transferred clonotypic CD4<sup>+</sup> T cells mediated pathology in C3-HA<sup>high</sup> mice and even induced lethality, it was of interest to determine whether they had been induced to become effector/memory cells or anergized. To address this issue, clonotypic CD4<sup>+</sup> T cells were recovered from the spleen 4 days after transfer (a time point before the onset of mortality) and analyzed for their ability to proliferate and secrete IL-2 in response to in vitro peptide stimulation. Because of the strong proliferation and expansion on encounter of the cognate Ag, 5% of the splenocytes of the C3-HA<sup>high</sup> recipient mice were 6.5 clonotypic CD4<sup>+</sup> T cells by day 4 posttransfer. There were far fewer 6.5 CD4<sup>+</sup> T cells in NT control recipients with transfer of the same number of naive 6.5 CD4<sup>+</sup> T cells (Fig. 4). These results suggested that the C3-HA<sup>high</sup> recipient mouse is an in vivo environment that induces tolerance of naive 6.5 clonotypic CD4<sup>+</sup> T cells. Although naive clonotypic CD4<sup>+</sup> T cells develop the capacity to mediate pulmonary vasculitis, with sufficient numbers even to cause lethality, they become tolerized in C3-HA<sup>high</sup> recipients.

Because pathology is mainly noted in the lung, we also checked the proliferative response of the lung-infiltrating lymphocytes retrieved 4 days after transfer, by restimulation in vitro with HA class II peptide-pulsed syngeneic splenocytes. A substantial proportion of the cells are clonotypic 6.4 T cells (up to 20%). They lost their Ag-specific proliferative response and IL-2 secretion as well (data not shown).
Clonotypic CD4+ T cells encountering cognate self-Ag express transient effector function before becoming tolerized

The paradoxical finding that adoptively transferred naive 6.5 CD4+ T cells caused autoimmune pneumonitis in C3-HA\textsuperscript{high} recipients while becoming anergic suggested that CD4+ T cells undergoing tolerance induction pass through an early phase with effector function. To examine this possibility more closely, we assessed the ability of the clonotypic CD4+ T cells to express two classic effector functions, i.e., cytokine production and provision of help to CD8+ T cells, during the initial phases of tolerance induction.

In the first experiment (Fig. 5), splenocytes were recovered from C3-HA\textsuperscript{high} recipients 2, 3, and 4 days after transfer and analyzed for their ability to proliferate, as well as to secrete IL-2 and IFN-γ, in response to in vitro restimulation with cognate HA class II peptide for 6.5 clonotypic T cells. Two days posttransfer, at the beginning of 6.5 clonal expansion, the splenocytes exhibited strong proliferative and IL-2 responses upon in vitro restimulation. They also secreted significant levels of the Th1 effector cytokine IFN-γ, although much less than that induced by vacc-HA (data not shown). These responses were transient. Even though the 6.5 clonotypic population continued to expand and their number increased through days 3 and 4 posttransfer, these responses to in vitro peptide restimulation diminished at day 3 and virtually absent by day 4. Because these experiments were performed by restimulating the bulk population of C3-HA\textsuperscript{high} transgenic splenocytes, it is difficult to exclude the effect of transgene-driven HA expression. Therefore, the clonotypic 6.5 CD4+ T cells were isolated by cell sorting from these C3-HA\textsuperscript{high} recipient mice also on 2, 3, and 4 days after transfer and restimulated in vitro with HA class II peptide-pulsed syngeneic NT splenocytes. The proliferative response and IL-2 and IFN-γ secretion of these purified cells also decreased with time (data not shown).

We next tested the ability of the clonotypic CD4+ T cells undergoing tolerance induction to provide help to cognate CD8+ T cells. In these experiments, C3-HA\textsuperscript{high} recipients received cotransfers of 2.5 \times 10^6 naive 6.5 clonotypic CD4+ T cells along with 2.5 \times 10^6 naive Clone-4 CD8+ TCR transgenic T cells that recognize a K\textsuperscript{b}-restricted HA epitope (7). When the HA-specific clonotypic CD8+ T cells were transferred by themselves to C3-HA\textsuperscript{high} mice, they underwent a slight clonal expansion in the spleen and a somewhat greater expansion in the lung-draining hilar LNs. However, when clonotypic CD4+ T cells were cotransferred with the clonotypic CD8+ T cells, the latter underwent greater clonal expansions in both the spleen and hilar LNs (Fig. 6). Inclusion of the clonotypic CD4+ T cells augmented the ability of the splenocytes retrieved 2 days posttransfer to proliferate in response to stimulation with the K\textsuperscript{b}-restricted HA peptide (Fig. 7A). This effect is even more significant if CD4+ T cells of the Clone-4 donor cells has been depleted (data not shown). CD8+ T cell production of IFN-γ elicited upon in vitro peptide stimulation was also dramatically enhanced by the CD4+ T cell cotransfer (Fig. 7B).

The critical effector functions derive from the naive clonotypic CD4+ T cells

The above data did not prove that the effector functions observed in our system were mediated by 6.5 CD4+ T cells that are truly microscopic pathology of the lungs of C3-HA\textsuperscript{high} recipient mice was examined 4 days after being transferred with different doses of 6.5 CD4+ T cells. The lungs of C3-HA\textsuperscript{high} mice transferred with 4 \times 10^5 and 2 \times 10^5 cells were normal without any perivascular infiltrates (not shown). The severity of perivascular cuffing (arrows) increased with the doses of cells, as shown here for 1 \times 10^5 (bottom), 5 \times 10^5 (center), and 2.5 \times 10^6 (top).
naive. To address this issue, we performed three separate sets of experiments. First, donor 6.5 T cells were sorted based on CD44 expression, the presumption being that clonotypic cells that had encountered cross-reactive Ags would be rendered CD44high. Thus 99.9% pure CD44low 6.5 clonotypic CD4+ T cells, from 0.8 × 10^6 to 2.5 × 10^6, were transferred into C3-HAhigh recipients. The results showed that CD44low cells were as capable as unsorted 6.5 cells (90–95% CD44low) in mediating mortality. On the contrary, transfer of an equal number of purely CD44high 6.5 cells did not result in recipient mortality. We further explored the hypothesis that naive cells mediate mortality by specifically transferring activated effector/memory 6.5 T cells. T cells were activated in vitro with HA class II peptide (1–10 μg/ml) before transfer to C3-HAhigh recipients. These in vitro activated 6.5 T cells were not able to mediate mortality. We also activated clonotypic 6.5 T cells in vivo by transferring the cells to NT B10.D2 recipients followed by activation with vacc-HA. Clonotypic cells were sorted and then transferred to C3-HAhigh recipients. Again, in vivo activated clonotypic donor cells were unable to mediate mortality. Together these data supported the hypothesis that the mortality in our system was mediated by naive T cells (Table I). Interestingly, although CD44high or activated 6.5 T cells did not mediate the mortality of C3HAhigh recipient mice, they expanded nicely in the recipients and still caused significant pulmonary pathology. There was perivascular round cell infiltration that was at least as prominent as those of the C3HAhigh mice that died from adoptive transfer of naive T cells. However, their alveolar space was relatively spared (Fig. 8).

**Discussion**

The major finding of the current study is that CD4+ T cells undergoing tolerance induction in response to encountering their cognate self-Ag express transient effector function before reaching a tolerant (i.e., nonresponsive) state. Thus, when naive TCR transgenic CD4+ T cells specific for I-Eα-restricted HA epitope were adoptively transferred into transgenic mice expressing high levels of HA, they were able to secrete the Th1 effector cytokine IFN-γ when restimulated in vitro with peptide pulsed APCs. Although this activity was strong when the T cells were recovered 2 days post adoptive transfer, it was virtually absent 4 days posttransfer. Additionally, the CD4+ T cells undergoing tolerance induction were able to provide help to CD8+ T cells that were also specific for self-HA. It has previously been shown that naive CD8+ T cells encountering self-Ag undergo an initial proliferative phase before reaching the development of tolerance (12–14). In our system, naive HA-specific CD8+ T cells also underwent an expansion phase subsequent to adoptive transfer into HA-expressing transgenic mice. Co-transfer of naive HA-specific CD4+ T cells resulted in even greater clonal expansion of the HA-specific CD8+ T cells, as well as the ability of these CD8+ T cells to secrete IFN-γ upon in vitro restimulation. This provides additional evidence that the CD4+ T cells expressed effector function before becoming tolerized. Perhaps the most significant example of effector function expressed by CD4+ T cells undergoing tolerance induction is their ability to mediate inflammatory autoimmune lung pathology in animals expressing high levels of HA and, with sufficient numbers, even to kill the recipient mice.

An inevitable limitation of all studies like this is that it is almost impossible to conclude that the CD4+ T cells that ultimately become anergic are the same T cells that acquire effector functions.
initially. Similar uncertainty still exists about the lineage of memory cells in the process of induction of effector/memory T cells and memory responses (15–17). In both cases, the effector cells that appear during the early phase of T cell activation may all be deleted, and the anergized or the memory T cells that emerge later come from a distinctly separate lineage. Instead of addressing this argument, we emphasize the fact that not every single CD4 T cell primed in vivo in a tolerogenic environment is anergized upon its first encounter with APCs. Most of the whole CD4 T cell population acquires a full-blown effector phase during the process of in vivo tolerance induction in our system.

In many TCR-transgenic systems, analysis of naive cells is often accomplished by crossing onto a recombination-activating gene (RAG) background to eliminate endogenous TCR expression that produces populations of memory cells. In the case of the 6.5 transgenics on a B6 or B10 background, extremely few transgenic T cells develop on a RAG background, and the few that do disappear rapidly when transferred into new recipients. We believed that this is because the 6.5 TCR is not efficiently selected positively in the B6/B10 background and requires a second TCR for positive selection. This does not render 6.5 mice a nonphysiological system because it appears that a significant proportion of normal T cells express two TCRs (18). There is also evidence that at least some of these dual TCR-expressing cells use one TCR for positive selection and the other for reactivity against a particular Ag (19, 20).

FIGURE 6. Before being anergized, 6.5 CD4 T cells help cognate Clone-4 CD8 T cells in expansion in vivo. Clone-4 T cells (2.5 x 106) were transferred into C3-HA high recipient mice with or without cotransfer of 2.5 x 106 6.5 T cells. Splenocytes and pulmonary hilar LN cells were harvested from 1 to 4 days after transfer. Clone-4 CD8 T cell clonotypic percentage was determined by flow cytometry stained with anti-CD8 and anti-Thy-1.1 Abs. Clone-4 CD8 T cells with cotransfer of 6.5 CD4 T cell displayed greater in vivo expansion than those without 6.5 cotransfer.

FIGURE 7. Before being anergized, 6.5 CD4 T cells prime cognate Clone-4 CD8 T cells in vivo to augment their in vitro proliferation and IFN-γ secretion. 2.5 x 106 Clone-4 T cells were transferred into C3-HA high recipient mice with or without cotransfer of 2.5 x 106 6.5 T cells. A, Splenocytes were harvested 2 days after transfer and stimulated in vitro with class I HA peptide. [3H]Thymidine was pulsed after 24 h of incubation for radioactivity counted another 16 h later. Ag-specific proliferation (left), IL-2 secretion (center), and IFN-γ secretion (right) were all lost gradually from day 2 to day 4. However, the 6.5 CD4 T cells from spleen did acquire an IL-2- and IFN-γ-secretory phenotype early after transfer.

FIGURE 5. Kinetics of proliferation and cytokine secretion for 6.5 CD4 T cells early after transfer into C3-HA high recipient mice. Splenocytes were harvested form C3-HA high recipient mice 2, 3, and 4 days after being transferred with 2.5 x 106 6.5 CD4 T cells. They were stimulated in vitro with MHC class II HA peptide. Forty-eight hours later, supernatant was harvested for ELISA cytokine analysis, and samples were pulsed with [3H]Thymidine for radioactivity counted another 16 h later. Ag-specific proliferation (left), IL-2 secretion (center), and IFN-γ secretion (right) were all lost gradually from day 2 to day 4. However, the 6.5 CD4 T cells from spleen did acquire an IL-2- and IFN-γ-secretory phenotype early after transfer.
The phenomenon we have reported has both similarities to and differences from the phenomenon of activation-induced nonresponsiveness (AINR). AINR was defined as nonresponsiveness of CD8+ T cells following full stimulation through both the TCR and costimulatory receptors (21). In contrast, the classical anergic state originally described for CD4+ T cell clones follows TCR engagement in the absence of costimulation (22–25). This developmental program of T cell activation is compatible with AINR of CD8+ T cells. However, the AINR CD8+ T cells originally described retain the ability to secrete IFN-γ upon TCR engagement (21, 26). Thus, AINR cells are similar to the classical anergic CD4+ T cell clones. On the contrary, the IFN-γ capacity of our CD4+ T cells is lost with development of nonresponsiveness.

This effector phase with in vivo tolerance induction is relatively efficient rather than merely abortive. Inflammatory tissue pathology was caused which is proportional to the number of clonotypic CD4+ T cells transferred, and in adequate numbers, these CD4+ T cells even mediate lethality of the transfer recipient mice. Although this is an artificial transgenic adoptive transfer system, we speculate a possible relevance to self-tolerance and autoimmunity.

Table I. Mortality of C3-HAhigh recipient mice by 7 days after adoptive transfer of different 6.5 T cell donor populations

<table>
<thead>
<tr>
<th>6.5 Donor Populations</th>
<th>No. of Cells Transferred</th>
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<tr>
<td></td>
<td>0.8 × 10⁶</td>
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<tr>
<td>Nonsorted (&gt;90% CD44low)</td>
<td>100% (6/6)</td>
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<tr>
<td>FACS sorted</td>
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</tr>
<tr>
<td>CD44low (&gt;99% CD44low)</td>
<td>100% (3/3)</td>
</tr>
<tr>
<td>CD44high (&lt;1% CD44low)</td>
<td>0% (0/3)</td>
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<tr>
<td>Effector/memory, generated</td>
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<tr>
<td>In vitro (&lt;10% CD44low)</td>
<td>0% (0/6)</td>
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<td>In vivo (&lt;10% CD44low)</td>
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*Neither the CD44bright subpopulation of 6.5 donor cells nor effector/memory 6.5 donor cells could cause recipient mortality.*

The phenomenon we have reported has both similarities to and differences from the phenomenon of activation-induced nonresponsiveness (AINR). AINR was defined as nonresponsiveness of CD8+ T cells following full stimulation through both the TCR and costimulatory receptors (21). In contrast, the classical anergic state originally described for CD4+ T cell clones follows TCR engagement in the absence of costimulation (22–25). This developmental program of T cell activation is compatible with AINR of CD8+ T cells. However, the AINR CD8+ T cells originally described retain the ability to secrete IFN-γ upon TCR engagement (21, 26). Thus, AINR cells are similar to the classical anergic CD4+ T cell clones. On the contrary, the IFN-γ capacity of our CD4+ T cells is lost with development of nonresponsiveness.

This effector phase with in vivo tolerance induction is relatively efficient rather than merely abortive. Inflammatory tissue pathology was caused which is proportional to the number of clonotypic CD4+ T cells transferred, and in adequate numbers, these CD4+ T cells even mediate lethality of the transfer recipient mice. Although this is an artificial transgenic adoptive transfer system, we speculate a possible relevance to self-tolerance and autoimmunity.

Because most of the described human or murine tumor Ags are nonmutated proteins also expressed in normal tissues, tumor immunity in many respects can be viewed as the flip side of autoimmunity, and the autoreactive T cell mediate beneficial antitumor effect. As we understand that there is an effector phase with tolerance induction by self-Ag, there should be antitumor activity before T cell tolerance by tumors. Similar results were observed in an analogous tumor system. As tumor specific CD4+ T cells were being anergized by a tumor Ag, they could nonetheless provide help for tumor specific CD8+ T cells in clearing lung metastasis (Z. Lu and H. I. Levitsky, unpublished data). In the context of tumor tolerance, this might actually explain the classical observation of concomitant tumor immunity. Even though the immune system may not eradicate large established tumors, new thymic emigrants of tumor-reactive T cells can be activated before they are tolerated and clear a smaller tumor implanted at the other site on that tumor-bearing animal. The implication for tumor immunotherapy is that, in addition to breaking tumor tolerance, how to sustain the antitumor activity before tolerance induction would be another feasible approach.

It has been previously reported that transfer of large numbers of OVA-specific CD8+ T cells results in diabetes and death in transgenic mice expressing OVA as a self-Ag in pancreas (13). Although this work implies effector functions with in vivo interaction...
of autoreactive CD8\(^+\) T cells and their cognate self-Ag, it is not clear whether the CD8\(^+\) T cells were anergized later. Even though we also observed an apparent effector phase before in vivo tolerance induction of HA-specific Clone-4 CD8\(^+\) T cells in our C3-HA transgenic system, there seems to be a couple of fundamental difference between CD4\(^+\) and CD8\(^+\) T cells. First, deletion plays a more important role in CD8\(^+\) tolerance. Deletion prevents the proliferating autoreactive CD8\(^+\) T cell population from expanding in a system with lower Ag concentrations in which the effector function is not detectable (29). Autoreactive CD8\(^+\) T cells do proliferate and expand and obtain effector functions in our C3-HA transgenic system, which probably contains higher Ag concentrations. Deletion is also required here to remove the cells with effector functions to make the whole CD8\(^+\) population tolerant (C.-T. Huang, Y. Yang, and D. Pardoll, manuscript in preparation). Second, the effector function of CD8\(^+\) T cells undergoing tolerance induction can be significantly enhanced with CD4\(^+\) T helper cells (Fig 7B).

The pulmonary pathology and lethality induced by these CD4\(^+\) T cells are directly proportional to the numbers that were adoptively transferred. Interestingly, we previously found that i.p. transfer of clonotypic CD4\(^+\) T cells into 3C-HA\(^{high}\) mice did not induce lethality (using the same number of cells that induce lethality when transferred i.v. in the current study) (5). Because the mice receiving i.p. transfers develop lung inflammation as do those receiving i.v. transfers (data not shown), it is likely that the lack of lethality associated with i.p. transfer simply results from slow diffusion of T cells from the peritoneal cavity into the bloodstream. Thus, i.p. transfer appears to have a similar effect to decreasing the number of transferred CD4\(^+\) T cells. In contrast, pulmonary pathology in 6.5 TCR- and HA-expressing double-transgenic mice further exemplifies the quantitative nature of our observation. Although adoptive transfer of naive 6.5 TCR transgenic T cells into 3C-HA\(^{low}\) mice does not cause any pathology, there is significant pulmonary vasculitis in double-transgenic mice generated by crossing 6.5 TCR to C3-HA\(^{low}\) HA-expressing transgenic mice (data not shown). Continuous thymic output of 6.5 CD4\(^+\)-transgenic T cells in these 6.5/C3-HA\(^{low}\)-double-transgenic mice is probably the cause of lung pathology.

These results are consistent with the concept that localization, dose, and time of availability of Ag are critical elements that determine the outcome of immune response (30). An interesting possibility raised by this notion is that CD4\(^+\) T cell tolerance results from chronic antigenic stimulation as previously suggested (31, 32). Thus, the tolerogenic APCs that present self-Ags (6, 33, 34) and immunogenic APCs that present pathogen-derived Ags (35–37) might deliver similar signals to the cognate naive CD4\(^+\) T cells which induces effector function. However, due to the constitutive and steady level presentation of self-Ags, compared with the potentially transient and fluctuating presentation of Ags deriving from rapidly cleared pathogens, effector function generated toward self-Ags might be attenuated due to T cell overstimulation. It has been shown recently that persistent presence of Ag, as a result of repeated vaccination, will not result in tolerance (38). Although it is not surprising that repeated vaccination does not result in tolerance, it does indicate that the simple presence of Ag alone is inadequate for T cell tolerance induction. Nonetheless, our data do not rule out the possibility that tolerogenic and immunogenic APCs deliver qualitatively different signals that commit cognate naive T cells toward differentiating into either tolerized or effector/memory cells, respectively, upon initial APC-T cell interaction.

This effector phase accompanied by tolerance induction might be organ specific. The different context in different organs could change the outcome of Ag encounter. Although the CD4\(^+\) T cells in effector phase caused vasculitis in the lung, actually there was no immune cell infiltrate in the other organs of high level HA expression in our C3-HA\(^{high}\) transgenic mice, i.e., the male reproductive organs.

This study demonstrates that CD4\(^+\) T cells undergoing peripheral tolerance induction express transient effector function before reaching a tolerant state, indicating that the tolerogenic and immunogenic CD4\(^+\) T cell differentiation pathways are more similar than previously thought. Thus, the main difference between the two pathways is not their ability to generate effectors, but rather their ability to sustain them.

References


