Direct Evidence That Functionally Impaired CD4+ T Cells Persist In Vivo Following Induction of Peripheral Tolerance

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Persistent Ag-Specific CD4+ T Cells Persist In Vivo Following Induction of Peripheral Tolerance

Kathryn A. Pape, Rebecca Merica, Anna Mondino, Alexander Khoruts, and Marc K. Jenkins

A small population of CD4+ T cells specific for self peptide/MHC molecules presented by thymic APCs are deleted in the thymus, although developing T cells specific for self peptide/MHC molecules derived from novel self proteins that appear in the blood after mature T cells have seeded the periphery (e.g., puberty- or pregnancy-associated hormones). These Ags would be accessed easily by naive self-reactive T cells because the peripheral blood and lymphoid tissues contain many class II MHC-expressing bone marrow-derived APCs that are capable of taking up, processing, and presenting Ags to circulating T cells. Therefore, it is reasonable to assume that the immune system must be able to silence the function of CD4+ T cells in the periphery. This has been well documented in studies in which soluble foreign Ags are introduced into the bloodstream. However, the mechanisms by which the T cells are silenced under these circumstances are not well understood.

Peripheral deletion in response to soluble Ag is one mechanism by which the immune system could eliminate self-reactive T cells that escape thymic deletion. Experimental models in which superantigens are injected into normal mice, or the relevant peptide Ags are injected into TCR transgenic mice, have provided evidence for peripheral deletion. However, in both of these model systems, a population of T cells survives. It is possible that this survival is artifactual because the abnormally high number of specific T cells present in these situations makes complete deletion impossible. Alternatively, deletion may be only an intermediate step in peripheral tolerance, and additional mechanisms may be required to fully silence self-reactive peripheral T cells.

Another potential mechanism for silencing T cells is functional inactivation, often referred to as anergy. Based largely on in vitro experiments with Th1 clones, anergy is defined as a defect in TCR-dependent proliferation that is acquired as a result of prior TCR stimulation in the absence of APC-derived costimulatory signals or proliferation (14–16). Although functionally unresponsive T cells have been identified in several in vivo systems (10, 12, 13, 17–25), the failure of a common induction pathway or molecular defect to emerge from these studies has raised doubts about the relationship between the in vivo models and the in vitro paradigm based on Th1 clones (26). Doubts about the relevance of the Th1 clone model have been reinforced by some studies in which naive T cells, the probable targets of peripheral tolerance in vivo, did not become unresponsive when stimulated through the TCR in the absence of costimulatory signals (27, 28). These issues have led some to argue that functional inactivation is not an important peripheral tolerance mechanism, or is only a brief stage that tolerized T cells pass through before deletion (29).

Recent studies have implicated immune deviation as an alternative explanation for the apparent functional unresponsiveness of CD4+ T cells in vivo. For example, it has been reported that what was thought to be anergy based on loss of Th1 lymphokine production is really skewing of self-reactive T cells, such that they respond to Ag only by producing a limited set of Th2 cytokines that are not capable of supporting T cell growth (30–32). It is also possible that in vivo functional unresponsiveness is due to Ag-specific suppression. It was reported recently that chronic activation of CD4+ T cells in the presence of IL-10 generates IL-10-, IL-5-, and TGF-β-secreting cells that suppress the proliferation of CD4+ T cells in vitro and prevent colitis in SCID mice (33).

We have attempted to address these mechanistic issues in detail by creating a situation in which a peptide/MHC-specific T cell population, large enough to be detected by flow cytometry following staining with an anti-clonotypic mAb, but small enough to behave in a physiologic manner when confronted with Ag in vivo, is monitored following the induction of peripheral tolerance. We demonstrated in a previous study that soluble Ag induces a transient accumulation and loss of adoptively transferred OVA-specific CD4+ TCR transgenic T cells that was accompanied by an

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apparent unresponsiveness in the surviving T cells (34). In this study, we found that a population of Ag-specific T cells, initially impaired in their ability to proliferate and produce lymphokines in vivo, survived for months after the initial Ag injection to the point in which they recovered from their unresponsive state. The unresponsive state was not associated with immune deviation or suppression. Therefore, tolerance to soluble Ag is maintained in part because the Ag-specific T cells that persist in the periphery possess an inherent activation defect during the time that the Ag is present.

Materials and Methods

**Mice**

DO11.10 BALB/c mice were produced by crossing the original DO11.10 TCR transgenic mice (35) for >10 generations to normal BALB/c mice. Offspring expressing the transgenic TCR were identified by flow-cytometric analysis of peripheral blood cells, as previously described (34). DO11.10 BALB/c mice were intercrossed, and offspring homozygous for the DO11.10 transgenes were identified by their ability to exclusively produce DO11.10 TCR-expressing offspring when crossed with BALB/c mice. DO11.10 BALB/c SCID mice were produced by crossing DO11.10 BALB/c mice for two generations with BALB/c SCID mice, and selecting offspring that contained DO11.10 T cells, but lacked B cells in the peripheral blood. The 6.5 TCR transgenic mice (36) were obtained from Dr. Hyam Levitsky (The Johns Hopkins University, Baltimore, MD). All TCR transgenic mice were housed in a pathogen-free facility according to National Institute of Health guidelines. Normal BALB/c mice, purchased from the National Cancer Institute (Frederick, MD) and housed in a conventional facility, were used as recipients of donor TCR transgenic cells.

**Injections and treatment of mice**

Lymph node and spleen cells (containing 2.5 × 10^6 to 5 × 10^6 CD4^+ KJ1-26^+ T cells) from TCR transgenic donors were prepared for adoptive transfer, as previously described (34), and injected into the tail veins of recipient mice in a volume of 0.3 ml of PBS. For the systemic Ag injections, OVA peptide 323–339 was dissolved in PBS and passed through a 0.22-μm syringe filter, and 300 μg was injected in a volume of 0.25 ml into the tail vein. For primary immunization, 30 or 300 μg of OVA peptide 323–339, or a mixture of 30 μg of OVA peptide 323–339 and 30 μg of hemagglutinin (HA)(1) peptide 111–119, was emulsified in CFA, and injected s.c. in a 0.1-ml vol into the tail or distributed between three sites on the back. For secondary challenge, 30 or 300 μg of OVA peptide was emulsified in IFA and injected s.c. in a 0.1-ml vol distributed between three sites on the back. The cell suspensions were injected intravenously (BrDU, Sigma, St. Louis, MO) was administered daily in the drinking water (0.8 mg/ml).

**Detection of TCR transgenic T cells**

Lymph node cells were first incubated in staining buffer (HBSS plus 2% FCS and 0.2% sodium azide) on ice with culture supernatant containing anti-FcR mAb (2.4G2; American Type Culture Collection, Rockville, MD) for 10 min to block FcR. Phycoerythrin (PE)-labeled anti-CD4 (PharMingen, San Diego, CA) and biotinylated KJ1-26 (37) or biotinylated 6.5 (36) mAbs were added and incubated for an additional 20 min. After a wash in staining buffer, the cells were incubated with FITC-labeled streptavidin (Caltag, South San Francisco, CA) for 20 min, washed, and analyzed on a Becton Dickinson FACScan flow cytometer. Twenty thousand events that had the light-scatter properties of lymphocytes were collected.

**Detection of cell surface activation markers**

Lymph node cells were incubated on ice with 2.4G2 culture supernatant for 10 min, followed by Cy-Chrome-labeled anti-CD4 mAb (PharMingen), biotinylated KJ1-26 mAb, FITC-labeled anti-CD45RB mAb, anti-L-selectin mAb, or anti-LEA-1 mAb (all from PharMingen). The cells were washed and incubated on ice for 20 min with PE-labeled streptavidin (Caltag). The FITC channel fluorescence of 1000 to 2000 CD4^+ KJ1-26^+ or CD4^+ KJ1-26^- cells was measured.

**Detection of BrDU incorporation**

Lymph node cells were stained with Cy-Chrome-labeled anti-CD4 mAb, biotinylated KJ1-26 mAb, and PE-labeled streptavidin, as described above.

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1 Abbreviations used in this paper: HA, hemagglutinin; BrDU, 5-bromo-2′-deoxyuridine; PE, phycoerythrin.
FIGURE 1. Some DO11.10 T cells persist and have an activated phenotype after OVA peptide injection. BALB/c recipients of DO11.10 T cells received nothing (A, B, C), an i.v. injection of 300 µg of OVA peptide (D, E, F), or an s.c. injection of 300 µg of OVA peptide emulsified in CFA on the back (G, H, I). Normal BALB/c mice that did not receive DO11.10 T cells were used as negative controls (J). Mice were sacrificed 12 (A, B, D, E, G, H) or 21 (C, F, I) days after the OVA peptide injections, and pooled brachial, axillary, and inguinal lymph node cells were stained with anti-CD4 and KJ1-26 mAbs. Some of the lymph node cells were stained with an anti-CD45RB mAb, an anti-BrDU mAb, or an isotype control mAb along with the anti-CD4 and KJ1-26 mAbs. In C, F, and I, the fluorescence intensity of CD4+, KJ1-26+ cells is shown.

The ability of soluble Ag to induce a hyporesponsive state in Ag-specific T cells was confirmed by measuring the capacity of DO11.10 T cells to accumulate in the lymph nodes following a secondary challenge with OVA peptide. Recipients that received no injection (naive), an i.v. injection of OVA peptide (tolerized), or an s.c. injection of OVA peptide emulsified in CFA (adjuvant primed) were given an s.c. injection of OVA peptide in IFA 12 to 21 days after the initial Ag injections. DO11.10 T cells accumulated dramatically in the draining lymph nodes of previously naive recipients, achieving a peak level on day 5 that was 30- to 60-fold greater than the starting level (Fig. 2A). In contrast, the DO11.10 T cells in tolerized recipients achieved a peak response on day 5 that was, at best, 10-fold greater than the level present on the day of challenge (Fig. 2A). The response in adjuvant-primed recipients was more difficult to interpret because the number of DO11.10 T cells present in the lymph nodes was at an elevated level at the time of challenge. In this case, the challenge injection caused DO11.10 T cells to accumulate maximally in the draining lymph nodes on day 3, an earlier time than in the naive or tolerized cases, but, like DO11.10 T cells from tolerized recipients, the cells only expanded 10-fold over the already elevated starting level (Fig. 2A).

populations of DO11.10 T cells that survived after day 12 in the OVA peptide-injected groups had responded to Ag in the past because they expressed low levels of CD45RB (Fig. 1, E and H) and, in BrDU-treated recipients, stained positive for BrDU (Fig. 1, F and I). This activated phenotype was not observed if the DO11.10 T cells were from recipients that did not receive Ag (Fig. 1, B and C), nor was it observed in the CD4+, KJ1-26+ cells of the recipients (Fig. 1, B, E, and H). Thus, a detectable, activated population of DO11.10 T cells persisted after a tolerizing injection of OVA peptide, indicating that deletion of all Ag-specific T cells is not the only tolerance mechanism operating in this system.

DO11.10 T cells from recipients injected previously with soluble Ag accumulate poorly in vivo following a subsequent antigenic challenge

The aforementioned experiments are complicated by the fact that a fraction of the KJ1-26+ cells present in the DO11.10 donor mice expresses a second TCR containing an endogenous Vα-chain due to inefficient allelic exclusion (41). Some of these dual TCR-expressing cells have a memory/activated phenotype (41), probably due to activation by environmental Ags. The coexistence of DO11.10 T cells with naive and memory phenotypes and more than one TCR is problematic for several reasons. First, it is difficult to exclude the possibility that a tolerizing injection of Ag deleted all of the initially naive DO11.10 T cells and the surviving population consisted of the double TCR-expressing cells. Second, it is possible that tolerance induction depended on interactions between the naive and memory DO11.10 T cells. To eliminate these possibilities, T cells from DO11.10 BALB/c SCID mice, which exclusively express the DO11.10 TCR and a naive phenotype (R. Merica, unpublished observation), were used for adoptive transfer. As shown in Figure 2B, a population of DO11.10 SCID T cells was clearly present in recipients injected 12 days earlier with soluble Ag, and these cells expressed less surface TCR than naive cells. Thus, it is unlikely that dual TCR-expressing DO11.10 T cells account for the surviving T cell population following tolerance induction. Furthermore, like normal DO11.10 T cells, DO11.10 SCID T cells in tolerized recipients expanded poorly in the draining lymph nodes following secondary challenge with OVA peptide in IFA (Fig. 2C), demonstrating that initially naive DO11.10 T cells can be tolerized in the absence of memory DO11.10 T cells.

The in vivo accumulation defect of tolerized DO11.10 T cells is reversible

In several other cases, peripheral T cell tolerance was reversed in the absence of the relevant Ag (17, 23, 42). To address this issue in our system, we examined the clonal expansion of DO11.10 T cells from tolerized recipients in response to Ag plus adjuvant challenge at various times after the initial i.v. injection of OVA peptide. As shown in Figure 3, A and B, DO11.10 T cells expanded poorly in response to a secondary challenge 12 to 23 days after the tolerizing injection of OVA peptide, as compared with DO11.10 T
cells from naive animals. In contrast, DO11.10 T cells from naive or tolerized recipients expanded similarly (Fig. 3, A and B) in response to Ag challenge 49 to 75 days after the tolerizing injection. However, the DO11.10 T cells remained in a hyporesponsive state in vivo if OVA peptide was injected once per week until challenge at day 49 (Fig. 3B). Thus, the persisting DO11.10 T cells recovered from the in vivo clonal expansion defect unless there was continuous administration of the Ag.

To conclude that the clonal expansion defect was correlated with the persistence of Ag, it was necessary to monitor the in vivo presence of the stimulatory OVA peptide/MHC molecules. A functional assay was devised because there are no reagents available for physical detection of this complex. Normal BALB/c mice were injected with OVA peptide. DO11.10 T cells were then transferred into these mice at later times. If OVA peptide/L-A^d complexes were present in a stimulatory form in the lymphoid tissues of the recipient, then the freshly transferred DO11.10 T cells should recognize these complexes and acquire the light-scatter properties of blasts. The sensitivity of this method is demonstrated by the finding that stimulatory OVA peptide/L-A^d complexes were detected in the lymph nodes of mice that were injected 41 days earlier with OVA peptide emulsified in IFA (Fig. 4A). As shown in Figure 4B, the DO11.10 T cells became blasts when transferred into mice, 3 or 5, but not 10 days after the mice received OVA peptide i.v. at the dose used in the tolerance experiments. Therefore, based on this assay, stimulatory OVA peptide/L-A^d complexes disappeared from the animals sometime between 5 and 10 days after i.v. injection of OVA peptide. Thus, disappearance of stimulatory OVA peptide/L-A^d complexes preceded reversal of the in vivo clonal expansion defect in the DO11.10 T cells, consistent with the idea that Ag persistence was required for maintenance of the hyporesponsive state.

Suboptimal accumulation of DO11.10 T cells from tolerized mice in response to Ag plus adjuvant priming is due to decreased proliferation

The failure of DO11.10 T cells from tolerized recipients to accumulate efficiently in lymphoid tissues following a second antigenic challenge could be related to decreased proliferation, as would be expected if they were functionally impaired, or to death of proliferating cells. To differentiate between these two possibilities, naive recipients, or recipients that were tolerized with one or two i.v. injections of OVA peptide were challenged 2 wk after the second i.v. injection with OVA peptide in CFA, and given BrDU until the time of sacrifice. If tolerized DO11.10 T cells proliferated in vivo at the same rate as naive DO11.10 T cells, but died at a higher rate, then the surviving cells from the two groups would be expected to have incorporated equal amounts of BrDU following Ag challenge. This was not the case, because 58 h after challenge, 73% of the DO11.10 T cells in previously naive recipients incorporated BrDU (Fig. 5A), whereas only 32.5 and 13.6% of the DO11.10 cells in recipients that were tolerized with one or two i.v. injections of OVA peptide, respectively, incorporated BrDU (Fig. 5, C and E). Therefore, the poor in vivo accumulation of DO11.10 T cells in the lymph nodes of tolerized mice correlated with reduced DNA synthesis. An increase in the frequency and total number of BrDU^+ DO11.10 T cells was observed in both tolerized and naive recipients 5 days after challenge (Fig. 5, B, D, and F), the time of peak DO11.10 T cell accumulation (Figs. 1, 2, and 5). However, the total number of BrDU^+ DO11.10 T cells present in tolerized mice was three- to sixfold lower than in naive recipients, consistent with a model in which the tolerized T cells do not proliferate as efficiently as their naive counterparts.

**DO11.10 T cells in tolerized mice have a cytokine production defect**

If the surviving DO11.10 T cells in tolerized recipients were functionally impaired, then the reduced proliferation of DO11.10 T cells in tolerized recipients following secondary challenge would be expected to be associated with reduced T cell growth factor production. Lymph node cells were taken from naive, tolerized, or adjuvant-primed recipients, 12 days after Ag injection, and assayed for IL-2 production following in vitro culture with OVA peptide (Fig. 6A) to address this issue. IL-2 production was mainly dependent on the presence of the transferred DO11.10 T cells,
since little or no IL-2 was detected if KJ1-26⁺ T cells were depleted from the lymph node cell suspensions before culture (Fig. 6A). This finding allowed for estimation of the amount of IL-2 produced per DO11.10 T cell present at the time of culture initiation (Fig. 6D). In the lymph node cultures from naive recipients, a significant amount of IL-2 was detected after the first 24 h, and the level consistently increased with each additional day of culture. Cultures from adjuvant-primed recipients contained the highest level of IL-2 after the first day, and the level of IL-2 did not increase thereafter, potentially due to consumption. Cultures from tolerized recipients differed, in that they did not contain detectable IL-2 after the first day, and contained 10- to 20-fold lower levels of IL-2 over the next 2 days than did cultures from naive recipients. In contrast, when lymph node cells were placed in culture 3 days after a tolerizing injection of OVA peptide (Fig. 6, B and E), the pattern of IL-2 production was identical to that observed in cultures from adjuvant-primed recipients. Finally, when lymph node cells were placed in culture 59 days after a tolerizing injection of OVA peptide, the pattern of IL-2 production in cultures from tolerized recipients was identical to that observed in cultures from naive recipients (Fig. 6, C and F). Little or no IL-2 was detected from any of the groups when the lymph node cells were cultured without Ag (data not shown). Together, these results show that Ag-specific T cells acquire an IL-2 production defect about 1 wk after i.v. injection of Ag, which is lost 8 wk later.

This conclusion was supported by experiments in which intracellular lymphokine staining was used to directly measure IL-2 production in vivo (Fig. 7). An i.v. injection of OVA peptide was used to facilitate synchronous Ag presentation during challenge. When naive recipients were injected with OVA peptide, about 15 and 30% of the DO11.10 T cells in the lymph nodes and spleen, respectively, contained intracellular IL-2 6 h later (Fig. 7A, lower left panel, and Fig. 7B). If CD4⁺, KJ1-26⁺ cells were examined (Fig. 7A, right panels, and Fig. 7B), or if the recipients were not challenged with Ag (Fig. 7A, upper left panel), less than 0.5% of the cells contained intracellular IL-2, demonstrating that IL-2 production could only be detected in activated DO11.10 T cells. Similar to DO11.10 T cells in naive recipients, 10 to 30% of the DO11.10 T cells in the lymph nodes and spleen from adjuvant-primed recipients expressed intracellular IL-2 following an in vivo
treated or previously given an i.v. injection of OVA peptide, suggesting that a suppressive environment was not created by the tolerizing Ag injection.

It was also possible that DO11.10 T cells from tolerized mice were secreting inhibitory factors, such as IL-10 and TGF-β, which have been shown to inhibit the proliferation of bystander T cells (33, 44, 45). To determine whether this was occurring, the ability of tolerized DO11.10 T cells to affect the in vivo behavior of a second, bystander population of 6.5 T cells transgenic T cells, which are specific for influenza HA peptide 111–119 bound to I-E^k class II MHC molecules (36), was monitored in vivo. BALB/c mice were injected with DO11.10 T cells.Recipient mice were then not treated or were injected with OVA peptide i.v. Twelve days later, the DO11.10 T cells in naive and tolerized mice were present at similar numbers in the lymph nodes (Fig. 9, A and C). Some of these recipients then were injected with 6.5 T cells, and the next day challenged with a mixture of the OVA and HA peptides in CFA. Tolerance was induced in the recipients of a previous OVA peptide i.v. injection, because DO11.10 T cells in these mice expanded only 10-fold, whereas DO11.10 T cells in naive recipients expanded 30- to 40-fold in the draining lymph nodes following the challenge injection (Fig. 9, B and C). However, the bystander population of 6.5 T cells expanded 30-fold in the absence or presence of naive or tolerized DO11.10 T cells (Fig. 9, B and C), suggesting that stimulation of tolerized DO11.10 T cells did not suppress the clonal expansion of a bystander population of 6.5 T cells present in the same lymph node. Identical results were obtained in an experiment in which the recipients were challenged with purified dendritic cells that were pulsed with a mixture of OVA and HA peptides, ensuring that the same APCs would be presenting Ag to both DO11.10 and 6.5 T cells (data not shown). The inability of tolerized DO11.10 T cells to suppress the proliferation of bystander 6.5 T cells was not explained by the fact that these T cells were incapable of being tolerized, because a prior i.v. injection of HA peptide into 6.5 T cell recipients resulted in poor in vivo clonal expansion (Fig. 10A) and poor in vitro IL-2 production (Fig. 10B) from 6.5 T cells upon secondary antigenic challenge. It was also notable that the presence of an expanding population of 6.5 T cells, which presumably would be producing IL-2, did not improve the clonal expansion of the tolerized DO11.10 T cells (Fig. 9, B and C), suggesting that a T cell growth factor response defect may be involved in the unresponsive state.

Discussion

Although previous studies on in vivo peripheral tolerance have implicated a role for clonal anergy, it was not clear whether the loss of clonal anergy induced in these studies was caused by an inherent defect in the responsive T cells or was merely an apparent defect truly related to cell death, immune deviation, or suppression. Much of the confusion in this area is related to the use of systems in which it was not possible to unambiguously determine whether the T cells bearing the appropriate TCRs are physically present, or the use of TCR transgenic or superantigen systems that contain abnormally high frequencies of Ag-specific T cells. We were able to address these limitations by physically tracking the in vivo fate of a small population of Ag-specific T cells that express a fixed TCR of known specificity, in normal mice subjected to a peripheral tolerance protocol. Furthermore, we relied almost exclusively on in vivo measures of T cell function to avoid in vitro artifacts.

Although most of the DO11.10 T cells that expanded following injection of adjuvant-free soluble Ag were rapidly depleted from the lymph nodes, probably due to apoptosis (9, 10, 46), a stable
population of DO11.10 T cells that displayed functional defects survived for at least 3 wk. In fact, a sizable population of DO11.10 T cells survived for several months after the Ag injection until they recovered their functional capability. However, the recovered DO11.10 T cells did not become activated at the late time points unless challenged with Ag probably because the relevant peptide/MHC molecules derived from the tolerizing Ag injection were no longer present. This finding highlights the fact that recovery of

FIGURE 5. BrDU incorporation following antigenic challenge is impaired in DO11.10 T cells from tolerized recipients. BALB/c recipients of DO11.10 T cells were left uninjected (A and B), given one i.v. injection of 300 μg of OVA peptide (C and D), or two i.v. injections of 300 μg of OVA peptide, 1 wk apart (E and F). Two weeks after the last injection, recipients were challenged with 300 μg of OVA peptide emulsified in IFA, and 58 or 120 h later, brachial, axillary, and inguinal lymph node cells were harvested and stained for detection of CD4 and the DO11.10 TCR, as described in Figure 1. Representative histograms of anti-BrDU mAb and isotype control mAb staining in CD4^+^, KJ1-26^+^ cells are shown along with the percentage of CD4^+^, KJ1-26^+^ cells that were BrDU^+^, the total number of cells that were CD4^+^, KJ1-26^+^, and the total number of CD4^+^, KJ1-26^+^ cells that were BrDU^+^ (mean ± SD for two to three animals/group) at each time point.

FIGURE 6. DO11.10 T cells from tolerized mice are defective in IL-2 production following in vitro stimulation. BALB/c recipients of DO11.10 T cells were left uninjected or were injected with OVA peptide, as described in Figure 1. Three (B and E), twelve (A and D), twenty-one, or fifty-nine days (C and F) later, brachial, axillary, inguinal, and cervical lymph node cells were harvested and stained with anti-CD4 and KJ1-26 mAbs. The remaining cells were placed in culture with irradiated BALB/c splenocytes, in the presence or absence of 5 μm of OVA peptide. In some cases, DO11.10 T cells were depleted with KJ1-26 mAb-coated magnetic beads before culture. The concentration of IL-2 measured in the supernatant from the Ag-stimulated wells is shown in A, B, and C. The frequency of CD4^+^, KJ1-26^+^ cells present in the lymph node cell suspensions was multiplied by the number of total lymph node cells/well to obtain the number of KJ1-26^+^ cells present at the time of culture initiation. The concentration of IL-2/well was then divided by the number of CD4^+^, KJ1-26^+^ cells present (D–F). The mean ± SD for five animals per group (A, B, D, and E) or two animals per group (C and F) is shown.
functional unresponsiveness by self Ag-reactive T cells would not necessarily lead to autoimmunity as long as the relevant self Ag is no longer expressed in the periphery.

An alternative explanation for why an unresponsive population of Ag-specific T cells would survive deletion is that the survivors possessed a preexisting activation defect, for example, due to low levels of adhesion molecules or a TCR mutation. This possibility was ruled out, however, by our finding that the majority of unresponsive DO11.10 T cells that persisted after i.v. Ag injection showed signs of prior activation.

The adoptive transfer system studied in this investigation provides direct evidence that not all Ag-specific CD4\(^+\) T cells are deleted during the induction of peripheral tolerance by soluble Ag. These findings argue against the idea that functionally impaired T cells are short-lived cells destined to die. A given T cell clone probably has the potential to react to multiple peptides presented in the context of a particular class II MHC molecule (47). Therefore, deleting a clone based on self-reactivity to one peptide would eliminate the ability of the immune system to use that clone to recognize other antigenic peptides. For example, if a single T cell clone was able to recognize both a peptide from human chorionic gonadotrophin, a hormone present only during pregnancy, and a peptide from influenza virus, deleting this T cell clone during pregnancy would partially compromise the ability of the immune system to respond to influenza virus. Thus, it would be advantageous if the immune system could simply silence a self-reactive T cell clone for the time that self Ag is expressed in the periphery.

DO11.10 T cells in tolerized animals did not accumulate efficiently in the lymph nodes following challenge with Ag. The finding that few BrDU\(^+\) DO11.10 T cells were detected 58 h after challenge suggests that a lack of proliferation, and not death of proliferating cells, is involved. However, the fact that the small number of DO11.10 T cells that accumulated in the lymph nodes 5 days after challenge were mostly BrDU\(^+\) suggests that tolerized DO11.10 T cells can proliferate to some extent in response to Ag. Because BrDU staining cannot detect the difference between one and multiple rounds of DNA synthesis, the presence of BrDU\(^+\) DO11.10 T cells following antigenic challenge of tolerized mice is consistent with the possibility that all of the DO11.10 T cells became hyporesponsive to some degree and underwent fewer rounds of DNA synthesis than naive cells. An alternative explanation would be that there was an outgrowth of a small subpopulation of DO11.10 T cells that escaped tolerance induction, and thus were able to proliferate normally in response to Ag. The double TCR-expressing memory cells from normal DO11.10 TCR transgenic

**FIGURE 7.** DO11.10 T cells from tolerized mice are defective in intracellular IL-2 and TNF-\(\alpha\) production following an in vivo antigenic challenge. BALB/c recipients of DO11.10 T cells were left untreated, or were given two i.v. injections of 300 \(\mu\)g of OVA peptide 1 wk apart, or an s.c. injection in the tail with 300 \(\mu\)g of OVA peptide emulsified in CFA. Two weeks after the last Ag injection, mice were challenged with an i.v. injection of 300 \(\mu\)g of OVA peptide. At the time of challenge, or 1.5 or 6 h after challenge, cells from brachial, axillary, inguinal, and cervical lymph nodes, or spleen, were immediately stained for detection of CD4, the DO11.10 TCR, and intracellular IL-2 or TNF-\(\alpha\). The amount of IL-2 staining in the CD4\(^+\), KJ1-26\(^-\) or CD4\(^+\), KJ1-26\(^+\) population is expressed as a dot plot, with a box identifying the percentage of cells staining positive for IL-2 (A). The mean percentage of cells staining positive for IL-2 or TNF-\(\alpha\) \pm SD was determined for two to five animals per group (B).

**FIGURE 8.** Intravenous injection of OVA peptide does not generate a suppressive environment in BALB/c mice. BALB/c mice were left uninjected or were given an i.v. injection of 300 \(\mu\)g of OVA peptide. Mice in both groups received DO11.10 T cells 11 days later. The following day, all recipients were challenged with an s.c. injection of 300 \(\mu\)g of OVA peptide emulsified in CFA in the back, and the percentage and total numbers of CD4\(^+\), KJ1-26\(^+\) cells in the brachial, axillary, and inguinal lymph nodes, at the indicated days, were determined as described in Figures 1 and 2. The mean \pm SD for two to three animals per group is shown. Similar results were obtained in a second experiment.
mice (41) are unlikely to account for a population that escaped tolerance induction, since residual proliferation following Ag challenge of tolerized recipients was also noted when DO11.10 SCID T cells were used for adoptive transfer. However, these studies have not eliminated the possibility that a small number of the transferred transgenic T cells do not encounter an OVA peptide/MHC-bearing APC during the period of tolerance induction, and thus remain in a responsive state.

The reduced capacity of DO11.10 T cells from tolerized mice to proliferate in response to antigenic stimulation was associated with impaired production of IL-2 and TNF-α, suggesting that an intrinsic defect in T cell growth factor production was responsible as in the case of Th1 clones (16). The DO11.10 T cells in tolerized mice expressed about twofold lower levels of TCR than DO11.10 T cells from naive mice. In addition, in some experiments the level of TCR expression increased as the T cells recovered their ability to respond to Ag (K. A. P., unpublished observation). It is therefore possible that the reduction in TCR expression was responsible for the functional defects. However, it is difficult to believe that this reduction is functionally significant given the low number of surface TCRs that need to be engaged for signaling to occur (48, 49). Indeed, our finding that in vivo persistence of Ag was required to maintain unresponsiveness suggests that the TCRs on tolerized DO11.10 T cells must be capable of transducing some signals. It is thus more likely that the tolerized DO11.10 T cells have a selective TCR-signaling defect, such that the signals required to maintain the unresponsive state are preserved, but those required for transcription of lymphokine genes are not. A precedent for this situation has recently been reported for unresponsive T cell clones, in which TCR-mediated calcium mobilization occurs, but activation of certain kinases does not (50–52).

**FIGURE 9.** DO11.10 T cells in tolerized recipients do not mediate bystander suppression. BALB/c recipients of DO11.10 T cells were left uninjected (DO11.10) or were given an i.v. injection of 300 μg of OVA peptide (DO11.10 tol). Twelve days later, some of the mice from each group received 6.5 T cells. The next day, mice were challenged with 30 μg of OVA peptide plus 30 μg of HA peptide emulsified in CFA. Brachial, axillary, and cervical lymph node cells were assessed for the percentage (A and B) and total numbers (C) of CD4+ KJ1-26+ cells or CD4+ 6.5+ cells, as described in Figures 1 and 2, on the day of challenge or 5 days after challenge. The mean ± SD for three animals per group is shown (n.a., not applicable). Similar results were obtained in a second experiment.
two animals/group). IL-2 was not detected in the supernatant of wells that
results from the recipients 12 days after the initial injections were placed in culture
numbers of 6.5 T cells were assessed as described in Figures 1 and 2 at the time of challenge (baseline), or 5 days after challenge, and the total
initial injections. Brachial, axillary, and inguinal lymph nodes were pooled
initiation. The concentration of IL-2 in the supernatant from Ag-stimulated
cells/well to obtain the number of 6.5 T cells present at the time of culture
vant-free soluble Ag and proliferate vigorously in the lymphoid
that DO11.10 T cells produce IL-2 early after injection of adju-
early broken by concomitant responses to irrelevant Ags.
lymph node suggests that this form of tolerance would not be eas-
factors provided by bystander 6.5 T cells responding in the same
clonal expansion defect of DO11.10 T cells within the same lymph nodes. Thus, there is no evidence
mice did not suppress the clonal expansion of activated bystander
maintain the putative suppressive environment, responded normally fol-
transferred into tolerized mice, which would be expected to con-
It was possible that the in vivo proliferation defect of the
DO11.10 T cells in tolerized mice was associated with immune
development or suppression. However, DO11.10 T cells from toler-
lized mice did not produce detectable levels of the Th2 cytokines, IL-4 and IL-5. Furthermore, naïve DO11.10 T cells that were
population of DO11.10 T cells present at the peak of clonal expansion
could be comprised of a predominant population of effector cells,
which are not unresponsive, and a minor subpopulation, which is
receptor expression of B7 molecules, which might give CD28 a competitive
advantage over the negative regulator CTLA-4 (59), allowing for
enhanced T cell clonal expansion instead of tolerance. Inflamma-
tion also induces cytokines (60–65) that promote T cell survival and
acquisition of the capacity to produce lymphokines such as
IL-4 and IFN-γ (66, 67). In the absence of inflammation, Ag would
be presented by APC expressing low levels of B7, a situation in
which only transient clonal expansion occurs and negative regu-
lation by CTLA-4 may generate T cells that have lost the ability to
produce IL-2 and have not gained the capacity to respond to
growth factors or produce IL-4 or IFN-γ. The necessity for
inflammation to produce the appropriate environment for effective
T cell responses would be a viable way to limit the function of T
cells specific for neo-self Ags that appear late in life and facilitate
the function of T cells specific for microbial Ags (29, 68), which
would always enter the system with an adjuvant.

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FIGURE 10. Intravenous injection of HA peptide induces a state of
functional unresponsiveness in adoptively transferred 6.5 T cells. BALB/c
recipients of 6.5 T cells were left uninject or given an i.v. injection of
300 μg of HA peptide. In A, the recipients were challenged with an s.c.
injection of 300 μg of HA peptide emulsified in IFA 12 days after the
initial injections. Brachial, axillary, and inguinal lymph nodes were pooled
at the time of challenge (baseline), or 5 days after challenge, and the total
numbers of 6.5 T cells were assessed as described in Figures 1 and 2
(mean ± range for two animals/group). In B, lymph node cells removed
from the recipients 12 days after the initial injections were placed in culture
for 24 h with irradiated BALB/c splenocytes, in the presence or absence of
5 μM of HA peptide. The frequency of 6.5 T cells present in the lymph
node cell suspensions was multiplied by the number of total lymph node
cells/well to obtain the number of 6.5 T cells present at the time of culture
initiation. The concentration of IL-2 in the supernatant from Ag-stimulated
wells was divided by the number of 6.5 T cells present (mean ± range for
two animals/group). IL-2 was not detected in the supernatant of wells that
were cultured in the absence of HA peptide.

It should be noted that the DO11.10 T cells did not manifest a
lymphokine production defect at early times after administration of
the tolerizing Ag. The time lag required for the defect to become
apparent could be explained by delayed production of a repressor
protein, as postulated for T cell clones (53). Alternatively, the pop-
ulation of DO11.10 T cells present at the peak of clonal expansion
could be comprised of a predominant population of effector cells,
which are not unresponsive, and a minor subpopulation, which is
already unresponsive. If the effector cells are short-lived, as pro-
posed by Swain et al. (54), and the unresponsive cells are long-
lived, then the unresponsive phenotype would only become appar-
ent as the effector cells died.

It was possible that the in vivo proliferation defect of the
DO11.10 T cells in tolerized mice was associated with immune
deviation or suppression. However, DO11.10 T cells from toler-
lized mice did not produce detectable levels of the Th2 cytokines,
IL-4 and IL-5. Furthermore, naïve DO11.10 T cells that were
transferred into tolerized mice, which would be expected to con-
tain the putative suppressive environment, responded normally fol-
lowing immunization. Finally, DO11.10 T cells from tolerized
mice did not suppress the clonal expansion of activated bystander
T cells within the same lymph nodes. Thus, there is no evidence
that suppressive mechanisms contribute to the defects in IL-2 pro-
duction and in vivo clonal expansion of the tolerized DO11.10 T
cells. In addition, the finding that the clonal expansion defect of
unresponsive DO11.10 T cells was not corrected by T cell growth
factors provided by bystander 6.5 T cells responding in the same
lymph node suggests that this form of tolerance would not be eas-
ily broken by concomitant responses to irrelevant Ags.

Results from several sources are now consistent with the idea
that the induction of peripheral T cell tolerance is dependent on an
initial phase of T cell activation (8–11, 34, 55). We have shown
that DO11.10 T cells produce IL-2 early after injection of adju-
vant-free soluble Ag and proliferate vigorously in the lymphoid
tissues for several days (34, 56). Abbas and coworkers recently
reported that blockade of the CD28-B7 pathway prevents this Ag-
dependent clonal expansion and results in phenotypically naïve T
cells that are fully responsive to antigenic stimulation at later times
(55). Furthermore, they showed that blockade of CTLA-4 con-
verted an Ag injection that normally induces tolerance into one
that induces priming. Together these results suggest that CD28-
dependent activation and CTLA-4 engagement are actually
required for in vivo peripheral tolerance to occur. This was not
predicted by the in vitro model, in which a lack of CD28 signaling
improves the induction of unresponsiveness in Th1 clones (57, 58).
Thus, although the unresponsive T cells that survive in tolerized
mice in vivo have some of the same lymphokine production de-
fects as anergic Th1 clones, these T cells may enter this state via
different mechanisms.

The DO11.10 T cells that survive in mice tolerized with Ag
alone, or primed with Ag plus adjuvant both show signs of prior
activation, and yet only the T cells from primed mice are rapid
and potent lymphokine producers. The simplest explanation for
the functional differences is that Ag presentation in the presence
of adjuvant-induced inflammation is required for naïve T cells to be-
come functional memory cells. Inflammation enhances APC ex-
pression of B7 molecules, which might give CD28 a competitive
advantage over the negative regulator CTLA-4 (59), allowing for
enhanced T cell clonal expansion instead of tolerance. Inflamma-
tion also induces cytokines (60–65) that promote T cell survival
and acquisition of the capacity to produce lymphokines such as
IL-4 and IFN-γ (66, 67). In the absence of inflammation, Ag would
be presented by APC expressing low levels of B7, a situation in
which only transient clonal expansion occurs and negative regu-
lation by CTLA-4 may generate T cells that have lost the ability to
produce IL-2 and have not gained the capacity to respond to
growth factors or produce IL-4 or IFN-γ. The necessity for
inflammation to produce the appropriate environment for effective
T cell responses would be a viable way to limit the function of T
cells specific for neo-self Ags that appear late in life and facilitate
the function of T cells specific for microbial Ags (29, 68), which
would always enter the system with an adjuvant.


