# Tetracycline-controllable Selection of CD4$^+$ T Cells: Half-Life and Survival Signals in the Absence of Major Histocompatibility Complex Class II Molecules

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## Abstract

A system that allows the study, in a gentle fashion, of the role of MHC molecules in naive T cell survival is described. Major histocompatibility complex class II–deficient mice were engineered to express Eα chains only in thymic epithelial cells in a tetracycline (tet)-controllable manner. This resulted in tet-responsive display of cell surface E complexes, positive selection of CD4$^+$8$^-$ thymocytes, and generation of a CD4$^+$ T cell compartment in a class II–barren periphery. Using this system, we have addressed two unresolved issues: the half-life of naive CD4$^+$ T cells in the absence of class II molecules (3–4 wk) and the early signaling events associated with class II molecule engagement by naive CD4$^+$ T cells (partial CD3 ζ chain phosphorylation and ZAP-70 association).

Key words: transgenic • lymph node • inducible expression • T lymphocyte

## Introduction

MHC molecules are crucial for the differentiation, survival, and function of T cells, a point most graphically illustrated by the phenotype of MHC-deficient mice (1–4). In the thymus, recognition of self-peptide–MHC complexes on stromal cells determines whether immature thymocytes die or live and mature into immunocompetent T cells (5). In the periphery, recognition of antigenic peptides presented by MHC molecules on the surfaces of APCs induces activation, proliferation, and differentiation into effector or memory cells. Another critical role of MHC molecules has only recently been recognized: they are essential for the maintenance of the peripheral T cell pool.

After emigrating from the thymus, naive T cells can survive in the periphery for extended periods in the absence of overt antigenic stimulation (6–10). A number of studies, using a variety of sophisticated techniques, have established that this persistence is dependent on continuous recognition of MHC class I or II molecules (11–17). For example, grafts of class II–positive thymi into class II–negative mice (11, 14) and virus-mediated transfer of class II genes into thymi of class II–deficient animals (12) was used to examine the role of MHC class II molecules on peripheral survival of naive CD4$^+$ T cells. Both strategies resulted in selection of CD4$^+$ thymocytes, but these cells emigrated into a periphery devoid of class II molecules, where they had an abbreviated half-life. Another strategy, entailing transfer of naive CD4$^+$ cells into hosts lacking appropriate class II molecules, yielded similar results (15). Parallel observations were made of the MHC class I–mediated survival of naive CD8$^+$ T cells (13, 16, 17). Experiments using monoclonal T cell populations further established that the survival signal provided to naive T cells could not originate from just any MHC molecule; rather, it required a specific allele, the same needed for positive selection in the thymus (13, 15).

Two important issues have been raised by these observations. One concerns the durability of naive T cell compartments in the absence of TCR–MHC interactions in the periphery. Some studies demonstrated substantial peripheral populations, decaying with half-lives on the order of a few weeks (11, 12). In others, the rate of decay was much quicker, a matter of days (13); yet others showed almost no peripheral T cells, even early in the experiment (14, 17). This variation could at least partially represent true biological differences, for example between CD4$^+$ and CD8$^+$ T cells or

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between T cells with high- or low-affinity TCRs. More prosaically, they could also stem from differences in the experimental systems, perhaps reflecting systematic artifacts. For example, several of the protocols involve surgical trauma; others involve perturbations imposed on lymphocytes by mechanical disruption of lymphoid organs in vitro before cell transfer. In addition, some of the procedures fail to adequately control for rejection phenomena, mediated by either natural killer cells or peculiar residual T cell populations (18–20). Thus, it is important to reexamine the half-life question in as innocuous a system as possible.

The second issue concerns precisely how MHC molecules are engaged by naive T cells and how the resulting signal promotes survival. The TCR is a likely candidate for interacting with MHC molecules, given the allele specificity described in some experiments, but it is conceivable that coreceptors are also involved or are even sufficient in some contexts. Indeed, it has recently been suggested that receptor/coreceptor corecognition of MHC class I molecules is required for naive $CD8^+$ T cell survival (21). It is also not known how the engagement of MHC molecules is relayed intracellularly and translates into a survival signal. Freshly isolated LN T cells do exhibit a detectable level of phosphorylated tyrosine residues in the TCR-associated CD3 ζ chain (22–25), and the tyrosine kinase ZAP-70 can be found in association with partially phosphorylated CD3-ζ, though in an inactive, unphosphorylated state (24, 26). Might these perturbations correspond to the survival signals induced by constitutive naive T cell interaction with MHC molecules?

To address these issues, we have exploited a noninvasive system that permits controllable selection of $CD4^+$ T cells, followed by their release into a class II–negative periphery. We adapted the binary tetracycline (tet)[1] control system pioneered by Bujard and colleagues (27, 28) to engineer mice that express MHC class II molecules in typically class II–positive cells in a tet-controllable fashion. The line employed here expresses Eα molecules in thymic epithelial cells only in the absence of tet. On the appropriate class II–deficient background, Eα expression leads to normal E complex display, $CD4^+$ T cell selection, and export into a class II–barren periphery. This system seemed ideal for tackling unresolved questions concerning MHC class II–mediated survival of naive $CD4^+$ T cells.

## Materials and Methods

### *Plasmids*

*CII-tTA.* The tTA coding region was excised from pUHD15-1 (29) as a 1,035-bp EcoRI–BamHI fragment and blunt end cloned into EcoRI-digested pDOI5, which provides an MHC class II promoter and the splicing and polyadenylation signals from rabbit β-globin (30). The resulting plasmid contained tTA flanked both 5′ and 3′ by intron sequences of the rabbit β-globin gene and under the control of the MHC class II $E\alpha^k$ promoter.

*Tet Operator Sequence Eα.* An 839-bp EcoRI fragment of $E\alpha^k$ cDNA (position 21–4,781 of the sequence in reference 31, with added RI sites) was subcloned into pKCR7 (32). The plasmid pUHD10-3 (33) was digested with PvuI and BamHI, and the resulting 1,089-bp fragment containing seven tet operator (TetO) sequences upstream of a minimal CMV promoter was subcloned into pKCR7 in lieu of its PvuI–BamHI fragment. The resulting vector contained the TetO, the CMV minimal promoter, and $E\alpha^k$ cDNA with a rabbit β-globin intron 5′ of this gene.

### *Mice*

CII-tTA segment (as a 4.5-kb BglI fragment) and TetO-Eα (as a 2.2-kb XhoI fragment) were injected into fertilized C57Bl/6 (B6) × SJL F2 eggs. The transgenes are routinely typed by Southern hybridization (transgenic [tg] mice were generated in B6 × SJL F1 mice according to standard procedures, and the resulting founder mice were analyzed by Southern hybridization or PCR [Eα or tTA sequence primers]). MHC class II–deficient ($A\beta^{o/o}$ and $E\alpha16.A\beta^{o/o}$) mice have been described (3, 34). For chimeras, recipient mice were irradiated 16 h before transfer (800 rads from a $^{60}Co$ source) and received $\sim 5 \times 10^6$ bone marrow cells injected intravenously and analyzed 6–8 wk after reconstitution.

Tet was administered in the drinking water (supplemented with 2% sucrose) at a concentration of 2 mg/ml (unless otherwise stated) for mice >3 wk of age and 0.2 mg/ml for mothers in breeding cages until weaning age (we verified that this concentration indeed inhibits expression in the offspring, via transplacental and milk transmission).

To follow the $CD4^+$ populations in individual animals, LNs were removed surgically as previously described (12). Up to a total of six data points were obtained from each mouse: the first from one inguinal node at the onset, then the contralateral inguinal, the left and right axillary, and then a popliteal LN. The final time point was taken from the remaining popliteal LN at the time mice were killed. Flow cytometry and immunohistology were performed as described (35).

### *RNA Analysis*

RNA was prepared and tested by S1 nuclease mapping as described (36).

### *Isolation of $CD4^+$ T Cells*

LN cells were incubated with mAbs to B220 (RA3-6B2), MHC class II (M5-114), and CD8 (anti-Lyt2, 53-6-7), followed by anti–rat Ig–coated magnetic beads (Dynabeads; Dynal) according to the manufacturer's instructions. The purity of the remaining $CD4^+$ cells was typically >97%, as assessed by flow cytometry. The remaining 3% consisted of cells that were negative for both CD3 and IgM.

### *Immunoprecipitation and Western Blot Analysis*

Purified $CD4^+$ T cells were washed several times in ice old PBS and lysed at $\sim 10^7$ cells/ml in 10 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100 with protease and phosphatase inhibitors. Lysates were incubated with mAbs to either TCR-ζ (6B10.2) or ZAP-70 (1E7.2) and protein G–Sepharose beads (Pharmacia) for 2 h at 4°C. Alternatively, lysates were incubated with antiphosphotyrosine-conjugated agarose beads for 4 h at 4°C. Immunoprecipitates were washed four times in lysis buffer containing 1% Triton X-100, boiled, and resolved on 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp.) or nitrocellulose (Schleicher & Schuell,

[1]*Abbreviations used in this paper:* RT, reverse transcriptase; tet, tetracycline; TetO, tet operator; tg, transgenic.

Inc.). After transfer, the membranes were blocked in 5% nonfat milk in 10 mM Tris/Cl, pH 7.5, and 0.5 M NaCl containing 0.1% Tween 20 and blotted with antiphosphotyrosine mAb (4G10), which was revealed with an enhanced chemiluminescence procedure as described previously (23). Western blots of anti–TCR-ζ immunoprecipitates were stripped and reprobed with anti–TCR-ζ mAb as described above.

*Calcium Responses*

LN cells were washed twice (RPMI, 5% FCS), resuspended at $10^7$ cells/ml in 5 μM INDO-1-AM (from a 1 mM stock solution; Molecular Probes, Inc.), and incubated for 30 min at 37°C. They were washed twice at room temperature, stained with anti-CD4–PE and anti-CD8–Tricolor (Caltag Labs.), washed, and brought to 37°C. An aliquot was run on the flow cytometer; anti-CD3 or anti-Vβ8 was added, and 1 min later, anti–rat IgG (10 μg per $10^6$ cells) was added and the cells were returned for analysis to the flow cytometer. Cells were maintained at 37°C throughout the analysis. Analysis was performed on a Coulter Elite fitted with a Coherent Innova 90.5 Argon laser with a 100 mW UV line at 351/363 nm. INDO-1 fluorescence was collected at 420 and 525 nm (20-nm band-pass filters; Coulter Immunology), and the intracellular $Ca^{2+}$ concentration was visualized as the 420/525 fluorescence ratio (37).

## Results

*Generation of Tet-responsive MHC Class II Tg Mice.* Mice expressing the Eα chain in a tet-controllable fashion were generated by combining two transgenes, one encoding a transcriptional activator and the other a reporter. This approach has been described previously (27, 28); the particular transgene combination we used is illustrated in Fig. 1. For the transactivator construct (CII-tTA), expression of the TetR-VP16 chimeric transactivator was placed under the control of the promoter/enhancer region of the murine Eα gene, known to be capable of driving expression of linked cDNAs in all the usual MHC class II–positive cells in mice (38). For the reporter construct (TetO-Eα), the Eα cDNA was put under the dictates of a CMV minimal promoter supplemented by seven TetO sequences, which serve as binding sites for TetR-VP16. In double-tg mice, the transactivator should be synthesized in class II–positive cells (thymic epithelial cells, dendritic cells, macrophages, B cells), where it should turn on transcription of the Eα gene; as the binding of TetR-VP16 to the TetO motif is inhibited by tet, introduction of tet should extinguish Eα gene transcription.

Tg mice were generated on the B6 × SJL background by independent microinjection of the transactivator and reporter constructs. Founder animals were obtained for CII-tTA and TetO-Eα, and double-tg mice were produced through cross-breeding. Unless otherwise stated, mice carrying both the transactivator and reporter constructs will be referred to as "transgenic" (tg). As the B6 and SJL strains carry a natural null mutation of the Eα locus (39), expression of the Eα transgene can be readily detected on this background, either as RNA or as an Eα–Eβ complex assembled in association with the endogenously encoded Eβ chain.

*Expression of Transgene-encoded Eα Chains.* Expression of the Eα reporter gene was examined in mice carrying just

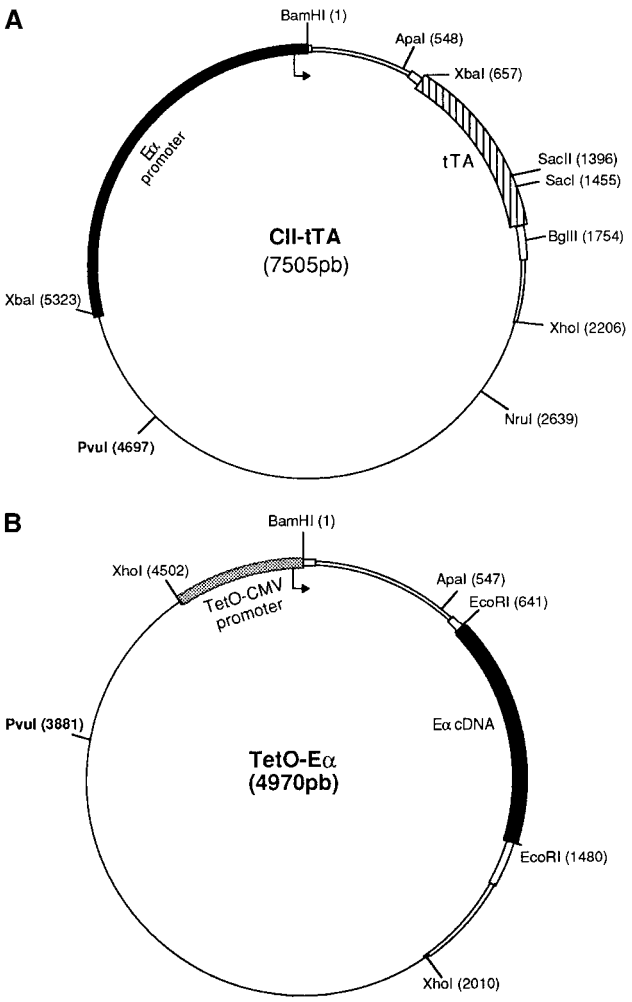


**Figure 1.** Construction of vectors for tet-responsive MHC class II expression. (A) The CII-tTA activator construct. The plasmid contains the MHC class II expression cassette of the pDOI5 plasmid (30) and contains the TetR-VP16 transactivator (tTA; hatched bar), Eα promoter/enhancer (solid bars), rabbit β-globin gene (double line), and plasmid backbone (single line). The numbers in parentheses correspond to the positions of the indicated restriction enzyme sites. (B) The TetO-Eα reporter construct. The plasmid contains the Eα cDNA (gray bar), seven TetO sequences, and a minimal CMV promoter (solid bar), rabbit β-globin gene (double line), and plasmid backbone (single line). The numbers in parentheses correspond to the positions of the indicated restriction enzyme sites.

the reporter transgene or both the transactivator and reporter constructs. In animals with only the reporter transgene, Eα transcripts were not detectable in any tissue examined by S1 nuclease analysis (Fig. 2 A, left). A complete absence of Eα mRNA was also seen by RT-PCR (not shown), confirming that there was no "leaky" expression of the reporter driven by the CMV minimal promoter. When both the reporter and transactivator constructs were present, substantial Eα transcripts were found in the thymus (Fig. 2 A, center). Interestingly, no Eα mRNA was detectable in the spleen or LNs or any other tissue examined, but it was clearly present in the thymi, spleens, and LNs of control B10.BR mice, as expected (Fig. 2 A, right). This restricted pattern of Eα transgene expression was also seen using RT-PCR

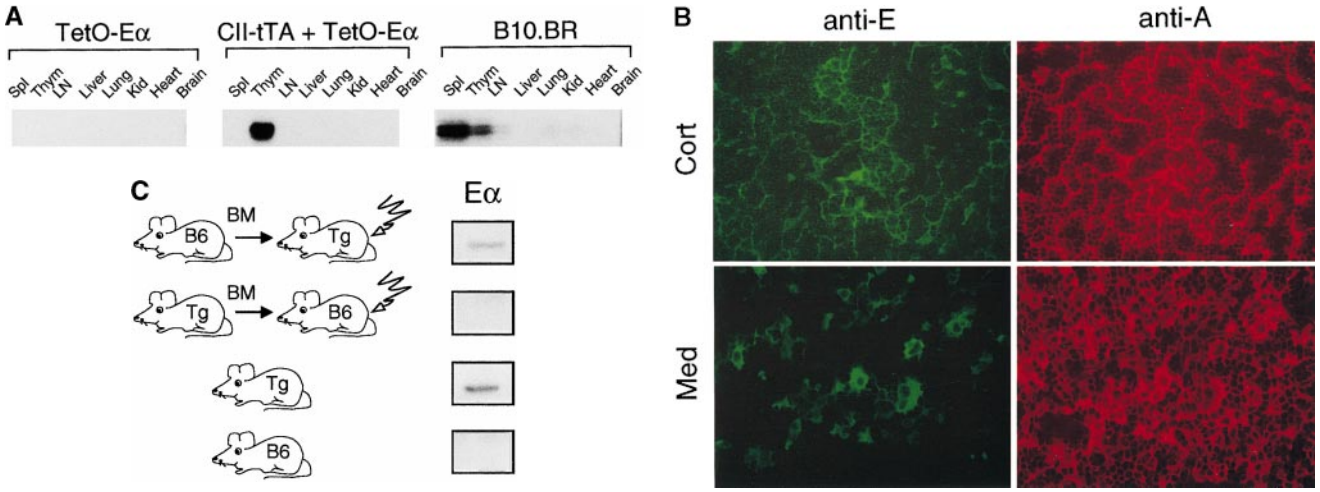


**Figure 2.** Distribution of tg MHC class II expression. (A) RNA was isolated from various tissues of mice containing the reporter construct alone (TetO-Eα), mice containing both the transactivator and reporter (CII-tTA + TetO-Eα), and control B10.BR mice. Eα mRNA was detected by S1 nuclease analysis. Spl, spleen; kid, kidney. (B) Thymus cryostat sections from a tg animal were stained with the mAbs 14.4.4 for E molecules (left panels) or Y3P for endogenous A molecules (right panels) and examined by fluorescence microscopy. (C) Eα expression in the thymus of reciprocal bone marrow chimeras between B6 and tg mice. Unmanipulated tg and B6 thymi are included as positive and negative controls, respectively.

(data not shown). Later analyses, via both S1 nuclease and RT-PCR, revealed that Eα mRNA was absent from the spleens and LNs of double-tg animals because the CII-tTA transactivator transgene was not transcribed in these tissues (not shown).

To monitor the expression of Eα protein (read out as E complex), we performed an immunofluorescence analysis of cryostat sections; costaining sections for endogenously encoded $A^b$ complexes served as an internal control. As expected from the RNA analysis, no E molecules were detectable in B or dendritic cells in the spleen and LNs. In contrast and as illustrated on the representative stains in Fig. 2 B, mAbs reactive to E and $A^b$ complexes showed essentially identical staining patterns in the cortex of the thymus. The intensity of E staining in the tg animals was comparable to that of control B10.BR mice. In the thymic medulla, however, a different picture emerged: although some medullary cells were E-positive, most A-expressing cells did not display E molecules. Immunofluorescent staining using a number of mAbs recognizing various medullary cell types, including ERTR5 (40) and 95 (41), established that the E-expressing cell type in the medulla was an epithelial cell (data not shown).

To obtain more information on the nature of the medullary cells expressing transgene-encoded Eα chains, we generated reciprocal bone marrow chimeras between regular B6 and tg mice. RT-PCR analysis of thymus RNA from chimeric animals demonstrated that only radioresistant stromal cells expressed the transgenes (Fig. 2 C). No Eα or tTA mRNA could be detected in the thymi of irradiated B6 mice that had received bone marrow from tg animals; conversely, irradiated tg hosts transferred with B6 bone marrow did make Eα transcripts. This dichotomy was confirmed by immunofluorescent staining of E complexes (not shown).

The transcriptional silence of the two transgenes in thymic cells of hematopoietic origin explains the "thymus only" expression pattern of Fig. 2 A: dendritic and B cells do not express the Eα reporter gene because they cannot transcribe the tTA transactivator gene. The reason for this restricted transcription pattern is unclear. The pDOI-5 vector, which donated the class II promoter/enhancer elements used to drive expression of the tTA construct, has been employed successfully to produce tg mice expressing a variety of cDNAs in all typically class II–positive cell types (30). On the other hand, problems with TetO transactivation in lymphoid or hematopoietic cells have been encountered in other contexts, even if surmountable. Cells of hematopoietic lineages may have difficulty expressing tTA, either because the prokaryotic coding sequences contain cell type–specific cryptic splice signals or because of an inherent toxicity in these particular cells.

In any case, CII-tTA/TetO-Eα mice should provide a valuable tool for the analysis of thymocyte differentiation and, in particular, of positive selection events, which rely on MHC molecules displayed by cortical epithelial cells.

*Tet-controllable Eα Expression.* Next, we established that expression of the Eα reporter was responsive to tet. First, an S1 nuclease analysis was performed on thymus RNA from pairs of tg mice that were or were not given tet in their drinking water for 7 d. As illustrated in Fig. 3 A, Eα mRNA was easily detected in samples from untreated mice, but no signal was seen after drug treatment. Indeed, the shut-off was so complete that transcripts could no longer be amplified by 35 cycles of RT-PCR (data not shown). It was also possible to completely block Eα mRNA expression in young mice by treating their mothers with tet during pregnancy and lactation (not shown).

How quickly Eα reporter expression could be turned off was examined by treating a group of adult tg mice with tet for various periods of time and measuring thymus RNA transcripts by S1 nuclease analysis. As shown in Fig. 3 B,

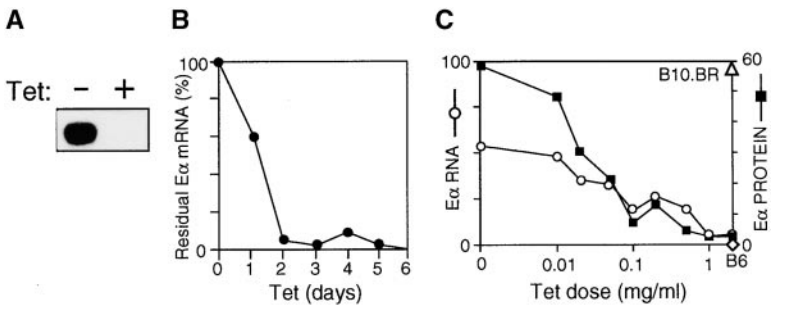


**Figure 3.** Transgene response to tet. (A) Tg mice were kept on 2 mg/ml tet in their drinking water (+) or not (−) for 7 d. RNA was extracted from thymi, and Eα expression was examined by S1 nuclease analysis. (B) Time course of tet response. Mice were treated for various times with 2 mg/ml of tet in their drinking water, and thymus RNA was analyzed as above. Eα expression was quantified by densitometry and is given as a percentage of maximum expression (without tet treatment). (C) Dose response to tet. Tg mice were treated with various doses of tet for 7 d. One thymic lobe was used for cryostat sections and quantification of Eα protein expression by staining with mAb 14.4.4 and analysis by confocal microscopy. RNA was extracted from the other lobe, and Eα mRNA was quantified as above. Values for protein are representative of staining intensity in the cortex and are given as arbitrary units; for RNA, values are normalized by comparison to RNA from a B10.BR mouse.

extinction of Eα mRNA was almost complete by day 2 of drug treatment. Loss of Eα protein, detected by anti–E complex immunofluorescence, was complete by 1 wk (not shown). It was possible to turn Eα transgene expression back on again by removing the drug; Eα mRNA levels fully recovered within a week with such protocols. Recovery was best when tet was removed after short treatment of adult mice; elimination of the drug subsequent to very long periods of treatment, starting prenatally, resulted in E complex expression patterns in the thymus that were somewhat "patchy" (not shown).

Treatment of tg animals with graded doses of tet resulted in partial reductions in Eα reporter gene expression (Fig. 3 C). Levels of both RNA (determined by S1 nuclease analysis) and protein (assessed by confocal microscopic quantitation of fluorescence intensity after staining thymus sections with an anti-E mAb) were reduced gradually by increasing the concentration of tet administered.

*Tet-controllable CD4⁺ T Cell Selection.* To simplify the analysis of $CD4^+$ T cell compartments, we crossed the CII-tTA and TetO-Eα transgenes onto the MHC class II–deficient Aβ° strain (3) (a combination hereafter abbreviated as tg/II°). Transgene-encoded Eα chains should pair with endogenously encoded Eβ chains in thymic cortical epithelial cells, and the resultant Eα–Eβ complexes restore positive selection of $CD4^+$ T cells (34). A prominent population of $CD4^+CD8^-$ thymocytes was detected in tg/II° mice in numbers equal to or even higher than those of wild-type littermates (Fig. 4, top panels). These cells appeared fully mature according to several phenotypic markers (e.g., CD69, peanut agglutinin receptor; not shown). They were exported to the periphery, with sizeable numbers of $CD4^+$ T cells populating the spleen and LNs (Fig. 4, bottom panels). However, the level of reconstitution in LNs was always slightly less complete than that in the thymus of the same mouse, the CD4/CD8 ratio in the former remaining below one. These cells presented all characteristics of conventional $CD4^+$ T cells selected by class II molecules: little expression of CD44 or CD69 activation markers and a broad repertoire of Vβ/Vα region usage without skewing toward a particular V element (data not shown).

When tg/II° mice were treated with tet from the prenatal period onward, mature $CD4^+$ T cell compartments were essentially absent from both the thymus and periphery (Fig. 4); only the minor CD1-selected population normally found in II° mice was present (18). Thus, the CII-tTA/TetO-Eα tg system permits investigators to manipulate at will the expression of MHC class II molecules on thymic epithelium and, consequently, the selection of $CD4^+$ T cells.

*Turnover of CD4⁺ T Cells in the Absence of Class II Molecules.* As documented above, transgene-encoded Eα molecules promote efficient selection of $CD4^+$ T cells, and these migrate to the periphery. Here they find themselves in a barren environment, devoid of MHC class II molecules, because expression of the Eα transgene is restricted to the thymus and because any residual expression can be shut off

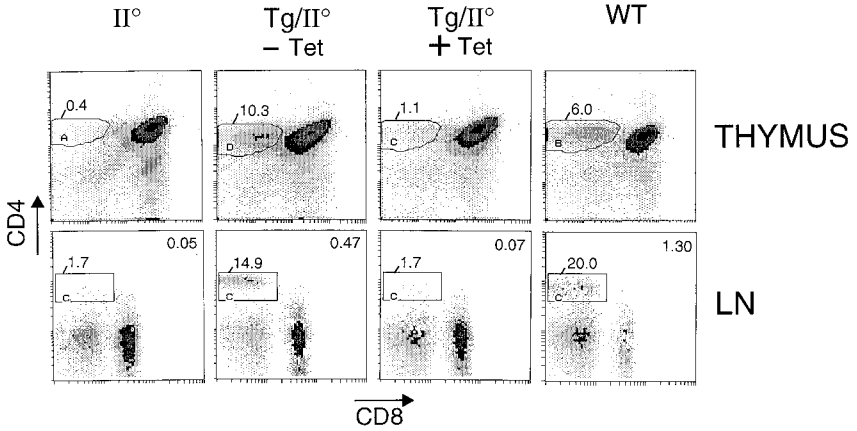


**Figure 4.** Selection of $CD4^+$ T cells on induced MHC class II molecules. CD4/CD8 profiles of thymus and LN cells of II° and wild-type (WT) mice or tg/II° mice that had either been left untreated (−Tet) or treated with tet (+Tet) from the prenatal period onward were analyzed by two-color cytofluorimetry. The percentage of $CD4^+$ cells in each panel is shown, and the CD4/CD8 ratio is indicated for LN. These profiles are representative of five or more such analyses.

by treating the mice with tet. This situation is similar to that created by thymus grafting or gene transfer into class II–deficient mice (11, 12, 14), but it is cleaner, free of surgical manipulations, and not clouded by caveats linked to rejection phenomena. Therefore, we have exploited this system to address outstanding questions related to $CD4^+$ T cell survival in the absence of class II molecules.

First, we assayed the turnover of $CD4^+$ T cells in such a context. In the experiments depicted in Fig. 5, adult tg/II° mice not previously treated with tet received a blocking dose of the antibiotic, thereby shutting down E complex expression in the thymus and positive selection of $CD4^+$ T cells. The proportion of $CD4^+$ cells in the periphery was measured by biopsy of individual subcutaneous LNs at various intervals thereafter. The levels were roughly stable for the first 2 wk, as expected because positively selected cells take 1–2 wk to complete their maturation and exit the thymus (12, 42). After this interval, a gradual decline of $CD4^+$ cells was observed, their proportion descending to levels equivalent to those found in class II–deficient mice by 10–15 wk after the start of tet treatment. This decline apparently followed unimodal exponential decay kinetics (Fig. 5, inset; $r = 0.92$, half-life 3.04 wk). The phenotypic characteristics of long-term survivors resembled the populations restricted by nonclassical class I molecules in II° mice, such as high levels of CD44 expression; data not shown). No such decline was observed after thymectomy of control B6 or Eα16.Aβ° mice (with or without tet treatment), which display A or E molecules, respectively, in their peripheral organs. We did observe a 1.5–2-fold drop in the proportion of $CD4^+$ cells after thymectomy in normal mice, but to levels that remained stable afterwards; this phenomenon has been described before and likely involves operative stress (7).

The results on mice with regulatable Eα expression indicate a half-life of ~3 wk for $CD4^+$ T cells in the absence of MHC class II molecules, a value strikingly similar to certain of those of some previous reports (11, 12).

*Signaling Molecule Changes in the Absence of Class II Molecules.* The CII-tTA/TetO-Eα transgenes in combination with the II° mutation provide a system in which large numbers of $CD4^+$ T cells are selected in the thymus and then released into a periphery devoid of MHC class II molecules. Biochemical analysis of signal transduction pathways can be performed during the decay of the $CD4^+$ compartment, revealing perturbations due to lack of class II molecule engagement. With normal naive T cells, TCR engagement by agonistic peptide/MHC ligands results in full phosphorylation of tyrosine residues in the CD3 subunits of the TCR, in particular in the CD3 ζ chain (25). Even in the absence of overt antigenic stimulation, freshly isolated mature thymocytes and naive LN T cells show constitutive phosphorylation of CD3-ζ tyrosines at a low level, with only one or two phosphorylated sites per molecule out of a possible six (22–25).

To compare the situation in a class II–barren environment, we isolated $CD4^+$ T cells from the LNs of tg/II° mice that had been under tet treatment for 2 wk, to ensure that the cells were mainly those that had emigrated from the thymus some time earlier and were soon to disappear. CD3 ζ chains were immunoprecipitated from lysates of isolated $CD4^+$ T cells, and their phosphorylation status was examined. As illustrated in Fig. 6 A, there was much less CD3 ζ chain phosphorylation in $CD4^+$ T cells from tet-treated tg/II° mice than in their counterparts from wild-type controls.

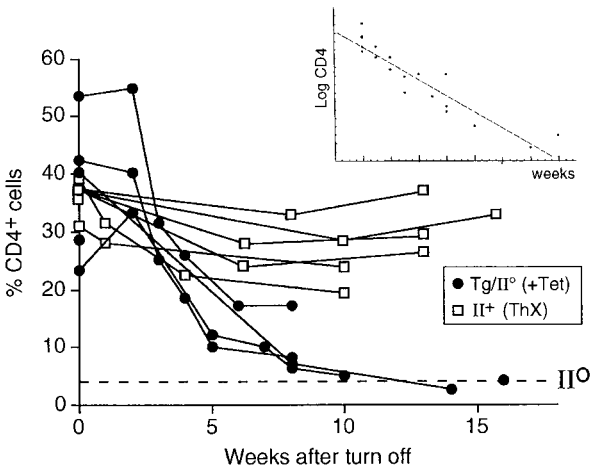


**Figure 5.** Decay of $CD4^+$ cells in LNs. The percentage of $CD4^+$ T cells in LNs of tet-treated tg/II° animals and thymectomized control $II^+$ mice was determined by flow cytometry after surgical removal of single LNs. ●, Tg/II° mice under treatment with tet; □, $II^+$ mice thymectomized at week 0 (these include B6 or Aβ°/Eα16 mice, some of which received tet). Dashed horizontal line represents the percentage of $CD4^+$ T cells in control II° animals.

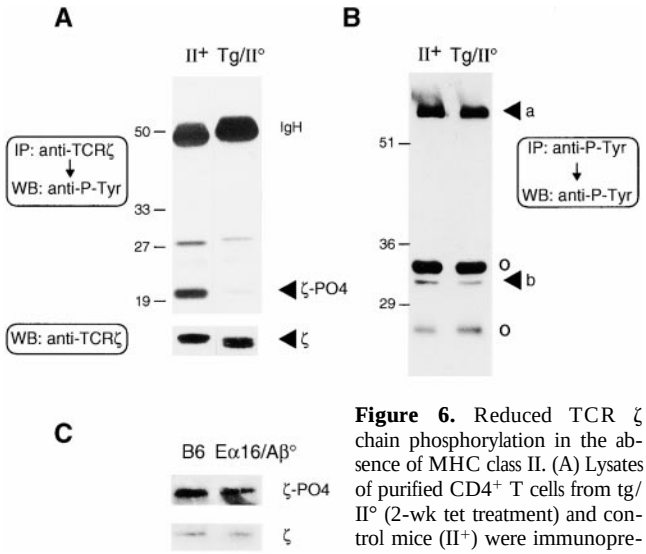


**Figure 6.** Reduced TCR ζ chain phosphorylation in the absence of MHC class II. (A) Lysates of purified $CD4^+$ T cells from tg/II° (2-wk tet treatment) and control mice ($II^+$) were immunoprecipitated with an anti–TCR-ζ mAb and probed sequentially on Western blots with antiphosphotyrosine mAb 4G10 (top panel) and anti–TCR-ζ mAb 6B10.2 (bottom panel). (B) The same lysates as in A were immunoprecipitated with antiphosphotyrosine-conjugated agarose beads. Immunoblots prepared from the eluted proteins were probed with antiphosphotyrosine, revealing bands labeled a and b. Background bands produced by Western blot of antiphosphotyrosine-conjugated agarose beads incubated without cell extract are indicated (O). (C) LN $CD4^+$ T cells from B6 and Eα16.Aβ° mice were analyzed as in A.

The difference corresponded to an ~10-fold reduction, whereas total amounts of precipitable ζ chains were equal (Fig. 6 A, bottom panel). Secondary immunoprecipitation and antiphosphotyrosine probing of lysates that had already been precipitated with anti–CD3-ζ indicated that the phosphorylation status of other proteins was not modified: $CD4^+$ cells from wild-type and tg/II° mice showed comparable phosphorylation levels for two other proteins, of ~32 and 55 kD (Fig. 6 B). Thus, diminished phosphorylation in the absence of class II molecule engagement seems to be a particular feature of CD3 ζ chains.

As the $CD4^+$ T cells from class II–positive ($II^+$) and tg/II° mice were selected on different MHC class II complexes A and E, respectively, it was possible that their variable CD3 ζ chain phosphorylation merely reflected the different selecting molecules. To evaluate this possibility, we compared tyrosine phosphorylation of CD3-ζ in $CD4^+$ cells isolated from a B6 and an Eα16.Aβ° mouse (the latter an Aβ° mouse complemented with a transgene expressing Eα in all class II–positive cells; reference 34.) CD3-ζ phosphorylation was the same in $CD4^+$ cells from the two types of mice (Fig. 6 C), confirming that the difference seen between $II^+$ and tg/II° animals was due to the absence of class II molecule engagement in the latter. This difference is not an artifact of tet treatment, as low levels of CD3-ζ phosphorylation were also seen in peripheral $CD4^+$ T cells of untreated tg/II° mice, and high levels have also been seen in tet-treated wild-type mice (not shown).

ZAP-70 is associated with tyrosine-phosphorylated CD3 ζ chains in freshly isolated LN T cells (24, 26). We wondered whether the reduced CD3-ζ phosphorylation seen in peripheral $CD4^+$ T cells unable to interact with class II molecules also resulted in reduced ZAP-70 recruitment. As indicated in Fig. 7, this was indeed the case. We were unable to coprecipitate phosphorylated ζ chains with ZAP-70 from lysates of freshly isolated $CD4^+$ T cells from tg/II° mice, whereas we could from the corresponding lysates from wild-type animals.

*T Cell Competence.* That $CD4^+$ T cells from tet-treated tg/II° mice had hypophosphorylated CD3 ζ chains with little ZAP-70 associated raised the question of whether they can respond to stimulation through the TCR. It is not possible to assay conventional T responses in these mice, as there are no class II molecules to present antigens. Instead, we evaluated a proximal measure of responsiveness, the $Ca^{2+}$ flux induced by cross-linking CD4 together with CD3 or the TCR, a strong stimulus that requires the integration of signals from receptor and coreceptor in proximity (43). The rate, extent, and duration of the $Ca^{2+}$ response to cross-linking was essentially the same for $CD4^+$ cells from tg/II° and control mice, whether anti-CD3 or anti-TCR (Vβ8) treatment was the stimulus (Fig. 8). Therefore, peripheral $CD4^+$ T cells that have not been in contact with MHC class II molecules can respond to stimulation via the TCR, indicating that cells lacking CD3-ζ phosphorylation and ZAP-70 association are not functionally inert.

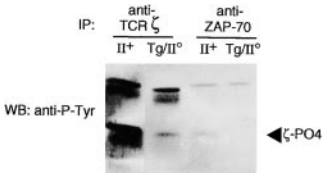


**Figure 7.** ZAP-70 association with phosphorylated ζ is reduced in the absence of MHC class II. Lysates of purified $CD4^+$ T cells were divided in two and immunoprecipitated with anti–TCR-ζ or anti–ZAP-70 mAb 1E7.2, and Western blots were probed with antiphosphotyrosine.

## Discussion

We have developed a system that permits regulatable positive selection of $CD4^+$ T cells. Class II–deficient mice were supplemented with a pair of transgenes that imparts tet-responsive expression of the Eα gene uniquely in thymic epithelial cells. In the presence of tet, the Eα gene is silent in both the thymus and periphery. In its absence, Eα transcripts are found in the thymus but nowhere else, Eα chains are synthesized and pair with endogenously encoded Eβ chains, and E complexes appear at the cell surface. Consequently, $CD4^+8^-$ thymocytes are efficiently selected and exit to the periphery. Several features of this system merit emphasis: (a) transcription of the Eα transgene strictly depends on the presence of the tTA transgene, as well as on the absence of tet (i.e., there is no "leaky" transcription); (b) E complexes are expressed at levels comparable to those of wild-type animals; and (c) E complex expression can be turned off and then back on again in a matter of days (with the exception of an uneven display when the reporter has been off for a long time from the earliest time of development).

While the system is "on," $CD4^+$ T cells are efficiently produced, and they emerge into a periphery barren of MHC class II molecules. Thus, tg/II° mice are valuable tools for studying the survival of naive $CD4^+$ T cells in the absence of class II molecule engagement. There is a powerful fail-safe mechanism for ensuring that the periphery is truly devoid of class II molecules: first, the Eα transgene is not expressed in the periphery of these animals—in particular, it is

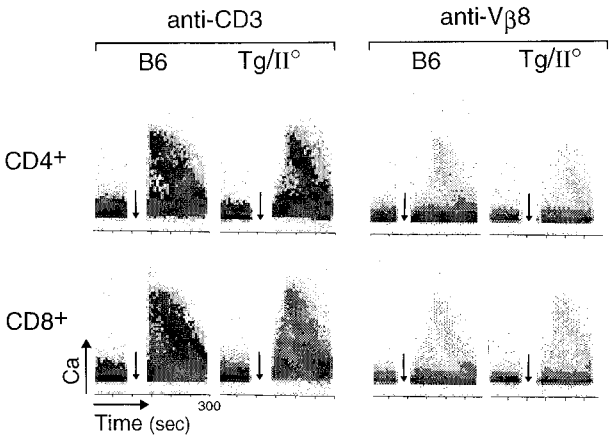


**Figure 8.** Cells devoid of TCR-ζ phosphorylation remain responsive to TCR signaling. $Ca^{2+}$ influx in LN cells was induced by the addition of anti–rat IgG to LN cells previously incubated with anti-CD3 or -TCR Vβ8, together with anti-CD4 and -CD8 (all rat mAbs). Intracellular $Ca^{2+}$ (INDO-1 fluorescence ratio) was monitored over time in gated $CD4^+$ and $CD8^+$ cells.

silent in hematopoietic cells (Fig. 2); second, turning the system "off" by adding tet eliminates the possibility that $CD4^+$ cells encounter class II–positive cells if they recirculate through the thymus or, conversely, that class II–positive cells exported from the thymus could "tickle" the $CD4^+$ cells. In contrast to experimental systems previously employed by ourselves and others (thymus grafts, intrathymic gene delivery, T cell transfer), the present strategy is noninvasive and merely requires supplementation of the drinking water with a harmless drug.

The first issue we addressed is the duration of naive T cell survival in the absence of MHC molecules. As mentioned above, widely variant estimates have been reported by several groups using diverse experimental strategies (11, 12, 14, 15, 17). It has been suggested that the longer half-lives of 3–4 wk estimated by Takeda et al. (11) and Rooke et al. (12) are artifactual, prolonged by engagement of class II molecules on dendritic cells exported from the thymus (44, 45). However, this was impossible in the latter case, because the adenovirus vector used to deliver class II genes to the thymus does not infect hematopoietic cells (12). Indeed, the half-life measured in our present study was also 3–4 wk. Engagement of class II molecules on dendritic cells exported from the thymus was also not a factor in these experiments, because the Eα transgene is only transcribed in thymic epithelial cells and, furthermore, all transcription was shut off by tet addition (Figs. 2 and 3). It remains to be seen whether the significantly shorter survival times reported for naive $CD8^+$ cells in the absence of class I molecules (13, 17) reflect relevant biological differences. That a CD4/CD8 subset dichotomy might be involved is supported by the recent suggestion that naive $CD4^+$ and $CD8^+$ T cells die by a different mechanism in the absence of peripheral MHC molecule engagement (21). It may also be relevant that one of the studies estimating a short half-life employed H-Y TCR–tg mice (13), and it has recently been demonstrated that the generation and function of T cells displaying different TCRs is variably dependent on signaling through phosphorylation of the CD3 ζ chain, H-Y T cells being very sensitive and others almost totally insensitive (46). Perhaps this variable dependence is reflected in variable survival times in the absence of TCR–MHC engagement and the ensuing CD3-ζ phosphorylation.

As it seemed likely that the TCR–coreceptor complex is somehow involved in the interaction with MHC molecules to promote naive T cell survival, we examined early events in TCR-dependent signal transduction pathways in the $CD4^+$ cells languishing in a class II–negative periphery. We found abnormally low levels of CD3 ζ chain phosphorylation (Fig. 6) and ZAP-70 recruitment (Fig. 7). CD3-ζ is a signal transduction molecule that carries multiple immunoreceptor tyrosine-phosphorylated activation motifs (ITAMs). The six ITAMs are heavily phosphorylated upon activation (25), recruiting nonreceptor tyrosine kinases of the ZAP-70/Syk family, which are consequently activated. Interestingly, CD3-ζ is constitutively phosphorylated, but only to a partial degree (pp21ζ), in naive T cells (22–25) and thymocytes (23, 47, 48) taken from normal mice. In these cells, pp21ζ is associated with ZAP-70, which, however, remains inactive (24, 26). The basal level of CD3-ζ phosphorylation has been attributed in large part to the src family kinase lck (49), and in T cell clones grown in vitro, pp21ζ correlates with the presence of active lck at the cell membrane (50).

Thus, it is likely that the basal phosphorylation of CD3-ζ to the pp21ζ form in primary $CD4^+$ T cells is the consequence of constitutive, weak TCR–MHC interactions, ultimately promoting survival. Unlike the fully phosphorylated pp23 form, pp21ζ would not transmit a full activation signal but would suffice for eliciting downstream survival events. A witness of this low-level signaling from pp21ζ may be the MHC-dependent, antigen-independent, low-intensity $Ca^{2+}$ flux that was observed in naive T cells pulsed with dendritic cells (51). Interestingly, pp21ζ does not occur in most T cell clones grown in vitro but is induced when they are stimulated by antagonistic peptides (52, 53). As most T cells in an animal are able to respond to antigenic stimuli, the implication is that the mode of signaling defined as "antagonistic" in vitro would actually correspond to cell survival signals in vivo, and we are led to question if and how antagonistic peptides introduced externally perturb this basal in vivo signaling.

In conclusion, the tg/II° system has provided a novel means to address in a noninvasive manner unresolved issues concerning naive T cell survival in the absence of MHC molecules. By coupling the CII-tTA transgene with other reporter transgenes, it will be possible to control at will the expression of other proteins in thymic epithelial cells and thereby to explore their roles in early as well as late T cell differentiation events.

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