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 Ea 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⁺ 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 CD3ζ 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 persistency 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. A nother 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
between T cells with high- or low-affinity TCRs. Moreover, 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 CD3ζ chain (22–25), and the tyrosine kinase ZAP-70 can be found in association with partially phosphorylated CD3ζ, 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) 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 EαK cDNA in thymic epithelial cells only in the absence of tet. On the appropriate class II–deficient background, EαK 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 EαK promoter.

**Tet Operator Sequence EαK.** An 839-bp EcoRI fragment of EαK cDNA (position 21–4,781 of the sequence in reference 31, with added R1 sites) was subcloned into pCR 7 (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 pCR 7 in lieu of its PvuI–BamHI fragment. The resulting vector contained the TetO, the CMV minimal promoter, and EαK cDNA with a rabbit β-globin intron 5' of this gene.

**Mice**

CII-tTA segment (as a 4.5-kb BglII fragment) and TetO–EαK (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. [EαK or tTA sequence primers]). MHC class II–deficient (Aβo/o and EαKβo/o) mice have been described (3, 34). For chimeras, recipient mice were irradiated 16 h before transfer (800 rads from a 60Co source) and received ~5 × 106 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 cold PBS and lysed at ~107 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,
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 mM 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 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 thymus, 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.
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Later analyses, via both S1 nuclease and RT-PCR, revealed that Ea 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 Ea protein (read out as E complex), we performed an immunofluorescence analysis of cryostat sections; costaining sections for endogenously encoded Ab 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 Ab 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 Ea 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 Ea 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 Ea 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 Ea 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 Ea Expression.** Next, we established that expression of the Ea 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, Ea 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 Ea transcripts. This dichotomy was confirmed by immunofluorescent staining of E complexes (not shown).
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 Eα 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 protein 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<sup>+</sup> T cell Selection. To simplify the analysis of CD4<sup>+</sup> T cell compartments, we crossed the CII-tTA and TetO-Eα transgenes onto the MHC class II–deficient Aβ<sup>−</sup> strain (3) (a combination hereafter abbreviated as tg/II<sup>−</sup>). 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<sup>+</sup> T cells (34). A prominent population of CD4<sup>+</sup>CD8<sup>−</sup> thymocytes was detected in tg/II<sup>−</sup> 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<sup>+</sup> 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<sup>+</sup> ratio in the former remaining below one. These cells presented all characteristics of conventional CD4<sup>+</sup> T cells selected by class II molecules: little expression of CD44 or CD69 activation markers and a broad repertoire of Vβ region usage without skewing toward a particular V element (data not shown).

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

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.

Figure 4. Selection of CD4<sup>+</sup> T cells on induced MHC class II molecules. CD4/CD8 profiles of thymus and LN cells of II<sup>−</sup> and wild-type (WT) mice or tg/II<sup>−</sup> mice that had either been left untreated (−Tet) or treated with tet (+Tet) from the prenatal period onward were analyzed by two-color cytfluorimetry. The percentage of CD4<sup>+</sup> cells in each panel is shown, and the CD4<sup>+</sup>/CD8<sup>+</sup> 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 sur-
cical 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 vari-
ous 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 thy-
mus (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 appar-
ently followed unimodal exponential decay kinetics (Fig. 5,
inset; r = 0.92, half-life 3.04 wk). The phenotypic charac-
teristics of long-term survivors resembled the populations
restricted by nonclassical 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.Αβ° 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 lev-
els that remained stable afterwards; this phenomenon has
been described before and likely involves operative stress (7).

The results on mice with regulatable Eα expression indi-
cate 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–TATA/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. Bio-
chemical analysis of signal transduction pathways can be
performed during the decay of the CD4+ compartment,
revealing perturbations due to lack of class II molecule en-

gagement. With normal naïve T cells, TCR engagement

by agonistic peptide/MHC ligands results in full phos-
phorylation 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 ma-
ture thymocytes and naïve 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 tet/II° mice that
had been under tet treatment for 2 wk, to ensure that the
cells were mainly those that had emigrated from the thy-
mus 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 Exam-
ined. 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 thymecto-

mized at week 0 (these include B6 or Aβ°/Ea16 mice, some of which re-
ceived tet). Dashed horizontal line represents the percentage of CD4+ T
cells in control II° animals.

**Figure 6.** Reduced TCR ζ chain phosphorylation in the ab-
sence of MHC class II. (A) Lysates of purified CD4+ T cells from tg/
II° (2-wk tet treatment) and control mice (II°) were immunopre-
cipitated with an anti–TCR-ζ mAb and probed sequentially on Westerm blots with antiphosphotyrosine
mAb 4G10 (top panel) and anti–TCR-ζ mAb 6810.2 (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 anti-
phosphotyrosine-conjugated agarose beads incubated without cell extract
are indicated (O). (C) LN CD4+ T cells from B6 and Ea16.Aβ° mice
were analyzed as in A.

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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 from 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 from II⁺ and tg/II° mice is to be assayed, as ZAP-70 association has been shown to be dependent on tyrosine-phosphorylated CD3-ζ. To test this possibility, we stimulated CD4+ cells from wild-type and II⁺ mice with anti-CD3 or -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.

**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 absence 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+ B cells 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 LTA 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 failsafe 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 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.

**Figure 8.** Cells devoid of TCR-ζ phosphorylation remain responsive to TCR signaling. Ca2+ 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 Ca2+ (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 Roke 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 CD3ζ 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 CD3ζ 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 CD3ζ chain phosphorylation (Fig. 6) and ZAP-70 recruitment (Fig. 7). CD3ζ 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, CD3ζ 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 CD3ζ 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 CD3ζ 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++ 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 tgll° 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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